# Properties and multiple molecular forms of rat brain acetylcholinesterase.

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# PROPERTIES AND MULTIPLE MOLECULAR FORMS OF RAT BRAIN ACETYLCHOLINESTERASE

Thesis submitted

by

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To Maria Jesus, my wife.

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# ABSTRACT

Solubilization procedures, kinetic and thermodynamic behaviour, separation of different molecular forms and estimation of their molecular weight have been achieved on rat brain acetylcholinesterase. It was found that most of the enzyme is bound to the membrane and about 15% is present in a soluble form. The remaining 85% was considered to be membrane-bound and to solubilise that form it was necessary to use Triton X-100 (1% w/v) when the enzyme was detached from the membrane almost quantitatively. When the enzyme was stored in dry toluene for periods longer than three months there was no significant increase of soluble enzyme without the detergent. Proteases treatment was not effective except in the case of papain when nearly 80% of the enzyme could be solubilised in one step with very low concentration of papain suspension and with a considerable increase in specific activity compared with triton solubilized enzyme.

All enzyme preparations studied showed inhibition by excess substrate and an optimum substrate concentration of 2 mM. The effect of different ions on the stability of the enzyme was also studied. The variation of Vmax and Km with temperature for the membrane, naturally soluble and the detergent solubilised enzyme was analysed by Arrhenius plots. The energies of activation were calculated and the significance of the transition temperatures, which were seen in some cases, was discussed.

Sucrose gradient centrifugation showed the presence of four

main forms with sedimentation coefficients of 16S, 10S, 6-7S and 4-5S. The four forms were present in most of the preparations but their relative contributions were quite different. Starch block electrophoresis and polyacrylamide gel electrophoresis showed up to five bands with different net charge while gradient gel electrophoresis revealed up to eleven forms with molecular weights in the range of 74,000-450,000. The effect of temperature, ions and mercaptoethanol on the electrophoresis patterns was also investigated.

Finally the enzyme was purified by affinity chromatography up to 300 times with a recovery higher than 60%. The stability of this partially purified preparation has been studied and experiments similar to those with the crude enzyme have been carried out.

# CONTENTS

# SECTION 1

## INTRODUCTION

"Veni Creator Spiritus
mentes tuorum visita"

# A. GENERAL BACKGROUND

## 1. History

From the beginning of this century, acetylcholine has been an important area of study for quite a lot of research workers. Hunt and Taveau (1906) discovered the powerful pharmacological effects of this drug which previously had been synthesized by Bayer in 1867. The theory of a 'chemical mediator' for neurotransmission was introduced by Elliot (1905) who suggested that some organic compounds could act as transmitters of the nervous impulse. Henry Dale (1913) demonstrated that a particular ergot extract when injected intravenously in cats produced a rapid but transient fall in blood pressure. He was particularly struck by the evanescence of this effect and with the fact that repeated doses of extract produced actions which were short-lived and which could be faithfully reproduced with successive doses. After these classic experiments, he predicted (1914) the possibility that the acetylcholine arising in the body might be rapidly hydrolysed by the tissues as to avoid detection.

A year later Hunt (1915) showed that small doses of physostigmine potentiated the effects of acetylcholine in the heart and blood vessels; and Führer (1917; 1918) established the same potentiation with acetylcholine induced contraction of frog's stomach and dorsal muscle of the leech. Führer (1918) actually suggested that physostigmine prolongs vagal bradycardia by inactivating an enzyme capable of splitting a neuro-humoral substance. The presence of such an enzyme in the small intestine of horse and pig was

indicated by the work of Abderhalden and Paffrath (1925) and in the following year Loewi and Navratil (1926) demonstrated that heart muscle extracts inactivated the "vagusstoff". This was interpreted as being due to the hydrolysis of a choline ester by an enzyme, the action of which was inhibited by physostigmine.

Subsequent studies have been concerned with the establishment of cholinesterases as a group of enzymes and their classification and function within the wider group of esterases.

## 2. Definition and Specificity

A large number of esters are hydrolysed by enzymes called, generically, esterases. The definition and further classification of cholinesterases has its origins in the discovery of Galehr and Plattner (1927) that serum and whole blood have different choline esters splitting activities. However, despite the previous contributions of Dale, Loewi and others, these workers explained the ACh inactivating effects of erythrocytes as being due to non-specific surface catalysis. Subsequently Engelhart and Loewi (1930) firmly established the existence of a choline-esterase in the red blood cell.

When it seemed quite reasonable to suppose that acetylcholine was a mediator of nervous activity, Stedman, Stedman and Easson (1932) were the first to ask if there was also a specific enzyme for the breakdown of acetylcholine. Establishment of this fact would have reinforced the idea that the enzyme's physiological function was to hydrolyse the ester and thus inactivate it. From horse serum they found an enzyme which hydrolysed only cholinesters but acted

on butyrylcholine faster than acetylcholine. This enzyme was called cholinesterase but Vahlquist (1935) showed that cholinesters were not hydrolysed exclusively by human plasma and Glick (1938, 1939, 1941) carried out an intensive investigation of the substrate specificities of this serum cholinesterase, showing the enzyme to have increasing activity with acylcholinesters as the chain length of the acid moiety is increased from $C_2$ to $C_4$ and decreasing activity thereafter. By substituting sulphur for oxygen in the ester linkage, enzymic activity was enhanced.

The first clear demonstration of the difference between human serum cholinesterase and the esterase in human erythrocytes was produced by Alles and Hawes (1940) who showed that the latter enzyme was inhibited by high concentrations of acetylcholine and in contrast to the serum enzyme, rapidly hydrolysed acetyl-β-methylcholine. Richter and Croft (1942), confirmed the differences between the two types of cholinesterase in blood by testing their properties with specific inhibitors. Later work by Mendel and co-workers (Mendel and Rudney, 1943 a,b; Mendel et al, 1943) on the differing sensitivities to inhibitors and substrates led to a general classification into true cholinesterase of the erythrocyte and pseudocholinesterase of serum. Zeller and Bisseger (1943) showed that the cholinesterase present in brain was similar to that in the erythrocytes. Nachmansohn and Rothenberg (1944,1945) while studying the enzyme in many types of conducting tissues showed that the behaviour of the enzyme towards various substrates was very similar in erythrocytes and conducting tissue but

quite different to the serum enzyme. The rate of hydrolysis of cholinesters by serum cholinesterase increased as follows: acetyl < propionyl < butyryl whereas the turnover number of cholinesterase in conducting tissue fell sharply for acyl groups larger than propionyl. Also the nature of the alcoholic group was found to be more critical for the activity of the serum cholinesterase than for that of conducting tissue. It was therefore apparent that there were fundamental differences between various esterases from different sources and attempts were made to provide a meaningful classification of the enzymes. The Mendel and Rudney classification was based on data which differed from that of other laboratories Nachmansohn and and Rothemberg (1944) classified the enzyme on whether the source was erythrocyte (specific acetylcholinesterase) or nervous tissue/ (acetylcholinesterase). However, these definitions are very general and Nachmansohn and Rothenberg themselves pointed out that although there was an esterase in erythocytes which was specific for acetylcholine this specificity was relative and unconditional.

On the other hand, it now appears that there is no clear cut classification of cholinesterases since types of an intermediate nature have been found. The plasma cholinesterase from rabbit, rat, cock, and probably frog, hydrolyses propionyl-choline most rapidly, whereas those of man, horse and dog hydrolyse butyrylcholine at the greatest rate. In contrast, the cholinesterase of a variety of fowl has most of the properties of a "propionylcholinesterase" but can hydrolyse acetyl-β-methylcholine (Augustinsson, 1971).

The plasma of teleostian fish and elasmobranchs provide a further example, since the enzyme is an acetylcholinesterase

on the basis of substrate specificity but differs from acetylcholinesterase in kinetic behaviour (Augustinsson, 1968). An even greater deviation from any general classification is found in the enzyme of plaice muscle (Pleuronectes platessa) which shows high activity with butyrylcholine but it is inhibited by excess substrate; a characteristic of an acetylcholinesterase (Lundin, 1967 a,b).

In order to distinguish cholinesterases from other esterases it is necessary to have a working definition and that of Augustinsson (1963) is probably as good as any: "Cholinesterases constitute a group of esterases which hydrolyse cholinesters at a higher rate than other esters, when hydrolysis rates are compared at optimum conditions regarding substrate concentration, pH, ionic strength, etc., using preparations free from other esterases. All esterases which show this specificity are inhibited by $10^{-5}$ M eserine". The Enzyme Commission of the International Union of Biochemistry (1964) has recommended that the cholinesterases be sub-classified into those which hydrolyse most rapidly butyrylcholine or propionylcholine (or their thio-analogs) and those which hydrolyse acetylcholine (or its thio-analog) most rapidly. The former, they proposed, should be designated cholinesterase (acylcholine acyl-hydrolase EC3. 1.1.8) and the latter acetylcholinesterase (acetylcholine hydrolase EC3. 1.1.7). However, even within the Enzyme Commission's classification of acetylcholinesterase, there can be anomalies. For example, Fahrney and Gold (1963) have showed that the enzyme from electric eel electroplax is not inhibited by phenylmethanesulphonyl fluoride but the enzyme from bovine erythrocyte is inhibited by this compound (Turini et al, 1969)

The general characteristics by which acetylcholinesterase may be distinguished from b.cholinesterase (bearing in mind the above exceptions) are summarised in Table I.1.

It was recognised by Aldridge (1953 a and b) and by Augustinsson (1958, 1959a,b,c) that certain esterases differ from cholinesterases. Aldridge (1953b) divided the serum esterases into two classes based on their sensitivity to inhibition by organophosphorus compounds: A - esterases are not inhibited, B- esterases are inhibited. The cholinesterases are B-esterases. Augustinsson (1959 a and b) used the designation A-esterases for arylesterases; B-esterases for aliesterases and called the cholinesterases C-esterases. The aliesterases are esterases which hydrolyse short-chain aliphatic carboxylesters other than cholinesters; most of these are resistant to inhibition by $10^{-5}$M eserine, but some are sensitive. Arylesterases hydrolyse aromatic esters at high rates and include most of the phosphorylphosphatases.

A further classification was introduced by Bergman and Rimon (1957). Under their scheme, A-esterases are not inhibited by organophosphorus compounds but react with them by hydrolysing them (phosphorylphosphatases); B-esterases are inhibited by organophosphorus compounds, and include the cholinesterases; and C-esterases are those which neither react with, nor are inhibited by, organophosphorus compounds.

Under the Enzyme Commission recommendations (1964) however, these divisions disappear and a far more rational nomenclature is proposed:

1. Carboxylesterases (E.C.3.1.1.1) for esterases which are sensitive to organophosphorus compounds but insensitive

to 10μM eserine.

2. Arylesterases (E.C.3.1.1.2) for esterases resistant to both eserine and organophosphorus compounds and which hydrolyse aromatic esters at high rates.

3. Acetylesterases (E.C.3.1.1.6) for esterases which act preferentially on acetic acid esters.

As pointed out by Augustinsson (1968) there is a very close relationship between carboxylesterases and cholinesterases and because both enzymes contain a serine residue in the active centre together with the similarities in enzymatic mechanism and different substrate specificity an interesting theory has been suggested for this, namely that both cholinesterases and carboxylesterases have evolved from a common serine containing enzyme. The serine hydroxyl group reacts with an ester to form an intermediary complex, acyl-enzyme complex, which subsequently reacts with an acyl receptor like water. It is this "esteratic site" in both enzymes which reacts with the organophosphorus compounds. During the evolution the cholinesterases have - according to Augustinsson - acquired the second binding site, the "anionic site". This enables the cholinesterases to bind preferentially cationic substrates such cholinesters. Augustinsson has demonstrated that the pseudo-cholinesterases of the propionyl type showed large species differences in several respects like specificity for cholinesters and sensitivity towards inhibitors, whereas the butyrylcholinesterases of different species were similar in these respects. Augustinsson concludes that mutational changes have produced these differences, the butyryl-cholinesterases being the more specialised form of

pseudocholinesterase. The active centres of acetyl and butyryl-cholinesterases are in all probability similar with respect to their "esteratic site". It seems that either their "anionic sites" (Augistinsson, 1968) or aminoacid residues surrounding them are different (Kabachnik et al, 1970) . It is clear that in the case of acetylcholinesterase, predominantly ionic, coulombic forces are active whereas in butyrylcholinesterase "Van der Waals" forces dominate at the "anionic site".

Kingsbury and Master (1970) have even proposed a possible phylogenetic development for all types of esterases from a common primitive esterase.

In addition to these fascinating theories, in a recent paper Vigny et al (1978), comparing the multiple molecular forms of acetylcholinesterase and butyrylcholinesterase from different rat tissues found that the protein showed different thermodynamic behaviour. There was also no interconversion between the two activities and no immunological cross-reaction between the two enzyme systems. These findings do not support the hypothesis of Koelle et al (1977 a, 1977b) according to which the specifities of both enzymes were obtained by some post-transcriptional modification of a single basic subunit without altering its participation in the different quaternary interaction which could result in the macromolecular heterogeneity of the enzyme. Vigny et al (1978) suggest that two different genes codify acetylcholinesterase and butyrylcholinesterase activities. They also suggest that the structural similarities between both enzymes which indicated cross-regulatory phenomena in between them, could imply that butyryl-

## TABLE I.1

## Properties and Nomenclature of Cholinesterases

| | Acetylcholinesterase (AChE) | Cholinesterase (ChE) |
|---|---|---|
| Systematic name | Acetylcholine hydrolase | Acylcholine acylhydrolase |
| E.C. number | 3.1.1.7. | 3.1.1.8. |
| Optimal substrate | Acetylcholine | Butyrylcholine (propionyl or benzoyl choline for some enzymes) |
| Effect of excess substrate | Inhibition | No inhibition |
| Utilisation of acetyl-β-methylcholine | Substrate | Non-substrate |
| Utilisation of butyryl- or benzoylcholine | Non-substrate | Substrates |
| Inhibition by BW62c47 | Strongly inhibited | Very weakly inhibited |
| Inhibition by ethopropazine | Weakly inhibited | Strongly inhibited |
| Optimum pH | 7.5-8 | 8.5 |
| Tissues and sources with high activity | Electric organ<br>Erythrocytes<br>Nerve tissue<br>Thymus | Serun<br>Pancreas<br>Heart<br>Liver |

In this thesis, the above nomenclature will be adhered to throughout with the abbreviations 'AChE' and 'ChE' referring to the individual enzymes and 'cholinesterases' used as a general term to cover both types of enzyme.

cholinesterase possesses a precise physiological rôle, closely related to that of acetylcholinesterase, but the multimolecular associations of these enzymes, which appeared to be exactly homologous, probably play an essential role in their function *in situ*.

## 3. Occurrence of the Cholinesterases

### a. Species and tissue distribution

Cholinesterases have been found in all the multicellular animals so far studied although characterisation by the selective use of substrates and inhibitors has not always been very rigorous. A number of reviews demonstrate the wide range of species which have been studied (Prosser, 1946; Augustinsson, 1948; Karczmar, 1963; Usdin, 1970) and an exhaustive list is neither profitable nor necessary. The ubiquity of AChE is demonstrated by its presence from the lowest groups of the animal kingdom such as the sea anemones, right through to the highest group, the mammals. The snail, Helix pomatia (Augustinsson, 1946), the ciliated mononucleated organism Tetrahymena gelii S (Seaman and Houlihan, 1951) and the sea anemone Sagartia (Augustinsson, 1948) all show acetylcholine hydrolysing activity. Generally, however, the highest concentrations of AChE are found within mammals. The exception to this is the very high level of AChE found in the electroplax of electric fish such as Electroforus Electricus and Torpedo marmorata (Marnay, 1937) and some snake venoms (Iyengar *et al*, 1938).

The plant kingdom also contains enzymes which catalyse the

hydrolysis of acetylcholine. AChE (Jaffe, 1970) and ChE (Jaffe, 1973) have both been identified in the mung bean. Fluck and Jaffe (1974) published a survey of sixty plant species containing AChE which includes some as familiar as potato and tomato.

The tissue localization was studied by Plattner and Hintner as early as 1930 but their pharmacological assay technique was not very satisfactory. More reliable work by Quastel et al (1936) and Glick et al (1939) established an extensive range of tissues which contain cholinesterase and in his comprehensive review Augustinsson (1948) lists the human tissues shown to contain cholinesterases. Only a few tissues seem to be devoid of the enzyme: saliva, gall bladder and bile (Augustisson, 1948). Gerebtzoff (1959) has also reported the absence in leucocytes.

b. Distribution and subcellular localisation in nervous tissue

Current theories on the localisation of cholinesterases have resulted from a wide variety of cytochemical, histochemical, ultramicroanalytical and centrifugation studies. A number of excellent reviews have dealt with the localisation in neuromuscular systems (Koelle, 1963, 1971; Friedenberg and Seligman, 1972; McMahan et al 1977), in peripheral nerves (Koelle, 1963, 1968; Gisiger, 1977), in the central nervous system (Silver, 1967; Nistri et al 1975) and during ontogenesis (Karczmar, 1963). As Koelle (1963) has pointed out: "there is probably no other enzyme, the cytological localisation of which has been so extensively described to date as acetylcholin - esterase". As a consequence of this extensive literature it is only possible to give a brief summary of the more important studies on

the cholinesterase in nervous tissue.

It is generally agreed that there are four known classes of cholinergic nerve fibres:

a. Somatic motor fibres to skeletal muscle

b. Preganglionic autonomic fibres to both sympathetic and parasympathetic ganglia

c. Parasympathetic and some sympathetic, post-ganglionic fibres to autonomic effector cells

d. Some, but not all, fibres of the central nervous system.

Koelle (1963) makes the general statement that known cholinergic neurons all contain high concentrations of acetylcholinesterase throughout their entire lengths, including the dendrites, perikarya, axon and axonal terminations. This has generally been substantiated by more recent results.

(i) Neuromuscular system (Fig. I.1). Marnay and Nachmansohn (1937, 1938) first showed that areas of the frog sartorius muscle which were innervated by motor nerves had an AChE activity up to six times higher than areas which were free from nerve endings. This finding was significant in that it suggested that AChE might play a special role at the myoneural junction, perhaps as a neuro-humoral transmitter. Knowing that the volume formed by the nerve endings at the myoneural junction was small, the actual concentration of enzyme appeared to be remarkably high. However, the reason for this was later discovered when it was found that the synaptic cleft between nerve and muscle was really a mass of infolding which allowed a much higher surface area to be presented for the hydrolysis

Fig.I.1a. Schematic drawing of a motor end plate as seen through a light microscope (from Couteaux, 1958)

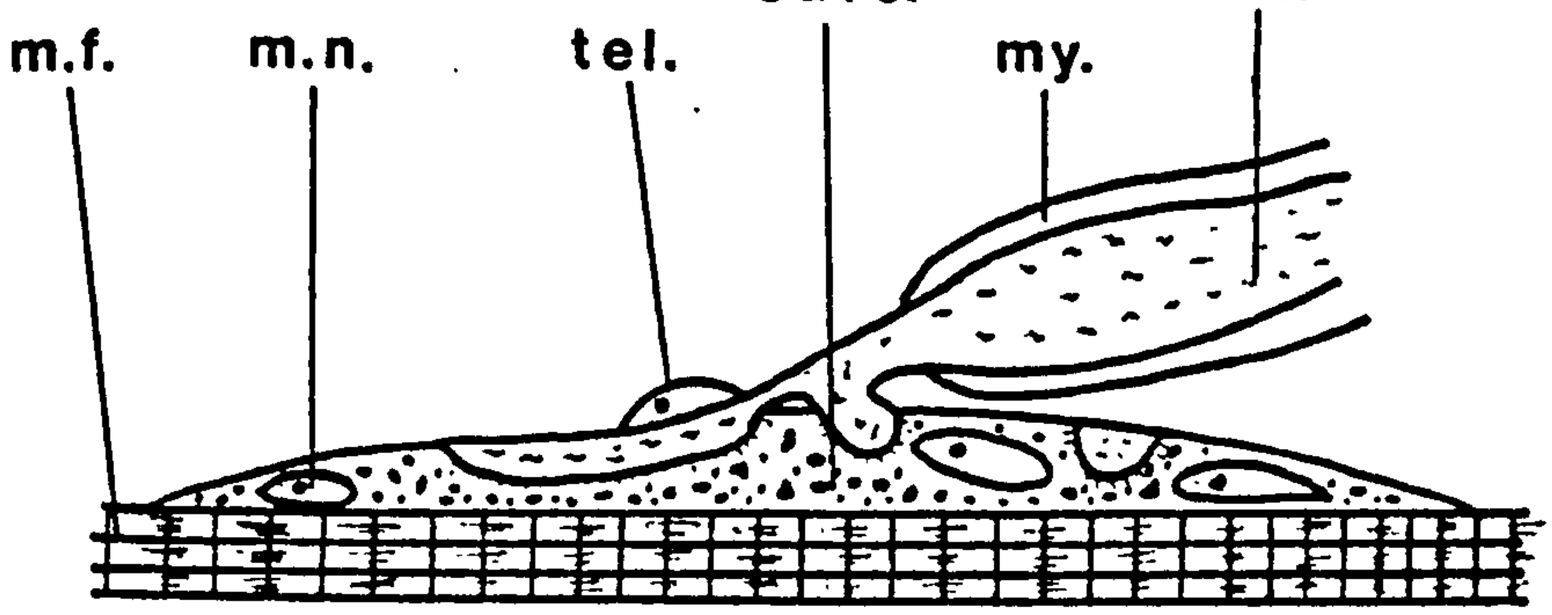



Fig. I.1b. Schematic drawing of a motor end plate as seen through an electron microscope (from Friedenberg and Seligman, 1972)

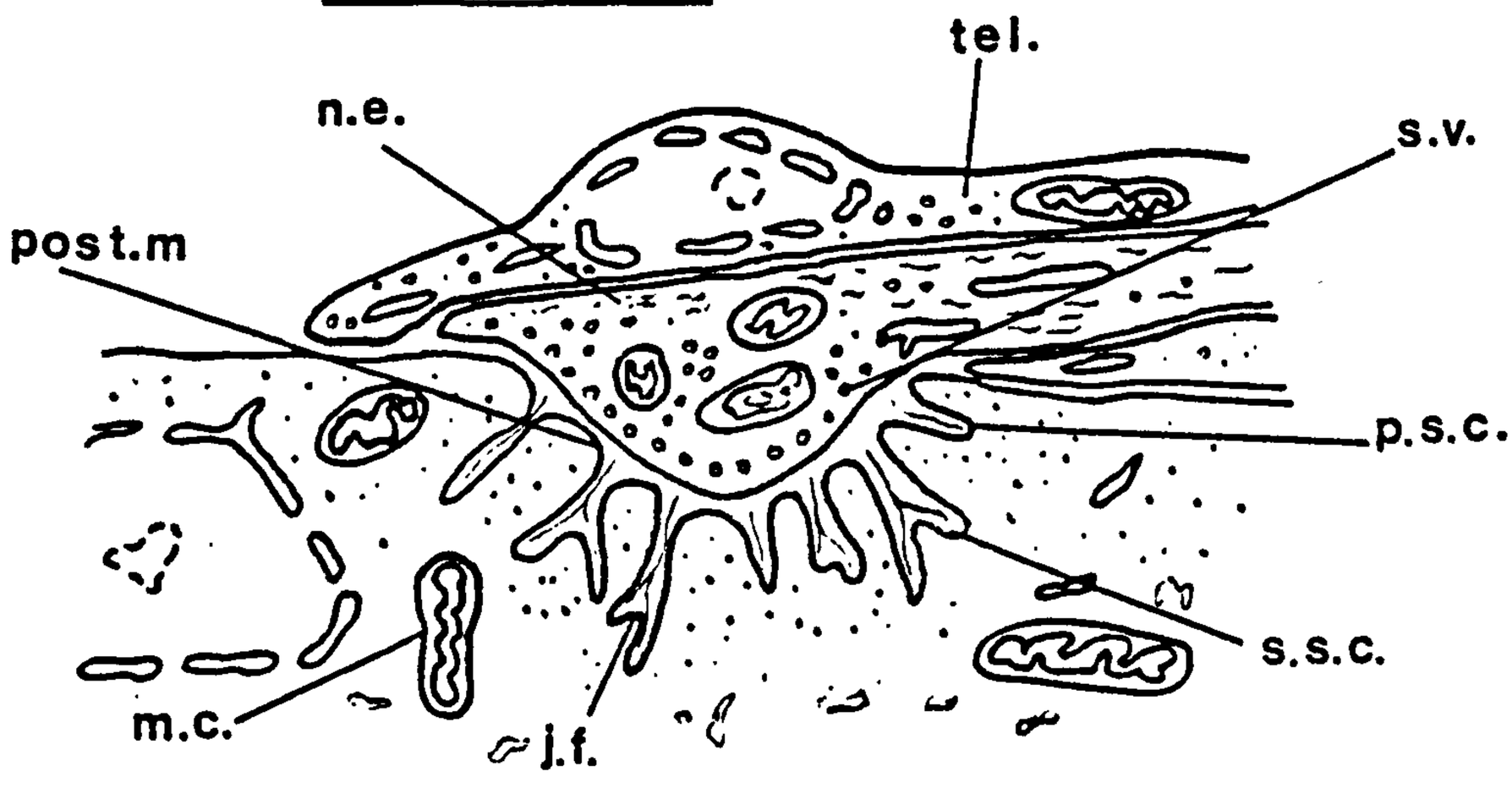



Fig. 1.1
Abbreviations: m.f., myofibrils; m.n., muscle nuclei; tel., teloglia (terminal Schwann cell); sarc., sarcoplasm; my. myelin sheath; ax., axoplasm; n.e., nerve ending; post.m., postsynaptic membrane; m.c., mitochondrion; j.f., junctional fold; p.s.c., primary synaptic cleft; s.s.c., secondary synaptic cleft; s.v., synaptic vesicles.

of ACh thanoriginally suspected. Karnovsky (1961), when studying the sarcoplasmic surface of rat skeletal muscle fibres, showed conclusively that AChE was present in high concentrations along the postjunctional membrane and it was suggested in that paper that the enzyme was synthesized locally. It was also shown by Couteaux (1955) and Salpeter (1967) that AChE is not confined to the end plate but is also present throughout the muscle fibres. Karnovsky (1964) demonstrated that in heart muscle ChE, not AChE, activity appeared in longitudinal elements of the sarcoplasmic reticulum, but not in the T - or transverse elements. He also found activity in the A-band, virtually no activity in the M-band or H-zone, and no activity in the Sarcolema or I-band. Terävänen (1969) extended this study to extraocular muscle and detected AChE in the T tubules while the other elements of the sarcoplasmic reticulum were negative.

In their review of the various histochemical methods used for detection of AChE at the motor end plate, Friedenberg and Seligman (1972) point out that the sensitivity and specificity of the method used dictates the results obtained. He quotes particularly the work of Adams, Bayliss and Grant (1969) working on nodes of Ranvier where it is argued that local staining in these regions might be due to the actual presence of AChE or alternatively the precipitation of thiocholine by copper in the stain binding to nodal acidic mucosubstances. This issue becomes particularly important when one looks into the problem of whether axonal transmission of the impulse is electrical or chemical in action.

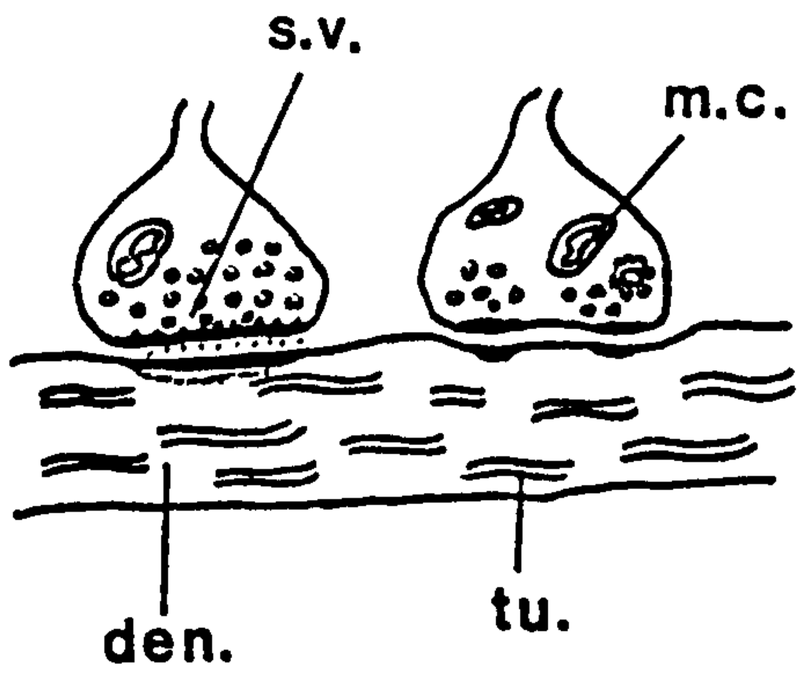
However, establishment of the fact that AChE is concentrated

at the myoneural junctions is consistent with Nachmansohn's (1970) neurohumoral transmission theory in which he argues that ACh release is intracellular causing permeability changes in the presynaptic and postsynaptic membrane rather than ACh actually crossing the postsynaptic membrane.

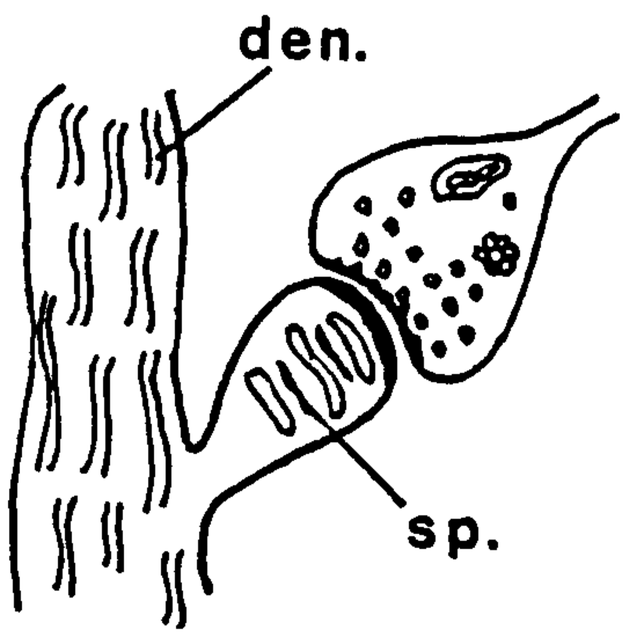
The results of Betz and Sakmann (1971) and Hall and Kelly (1971) open up the whole question of the true localization of the enzyme. Both sets of workers treated neuromuscular junctions with collagenase and removed the "ectolemmal sheath" (Betz and Sakmann, 1971) which they suggest acts as a "cement" substance between nerve and muscle. Since the AChE activity disappears with this sheath, these workers postulate that the enzyme is not a structural component and this is born out by the fact that in non-innervated portions of muscle the enzyme is not released by the collagenase treatment.

(ii) Autonomic nervous system (Fig. I.2). When it was found that AChE totally disappeared following preganglionic denervation, it was concluded that the enzyme was confined exclusively to the pre-synaptic terminals (Koelle and Koelle 1959). The hypothesis was therefore mooted that ACh released in preganglionic synapses at the presynaptic side, amplifies further ACh release by a positive feed back, and this is modulated by the AChE (Koelle, 1962). This fits in with the theory (Burn and Rand, 1959) that ACh facilitates the release of other transmitters at some non cholinergic nerve endings and might explain the presence of high levels of AChE in parts of the adrenergic nervous system (Koelle, 1971).

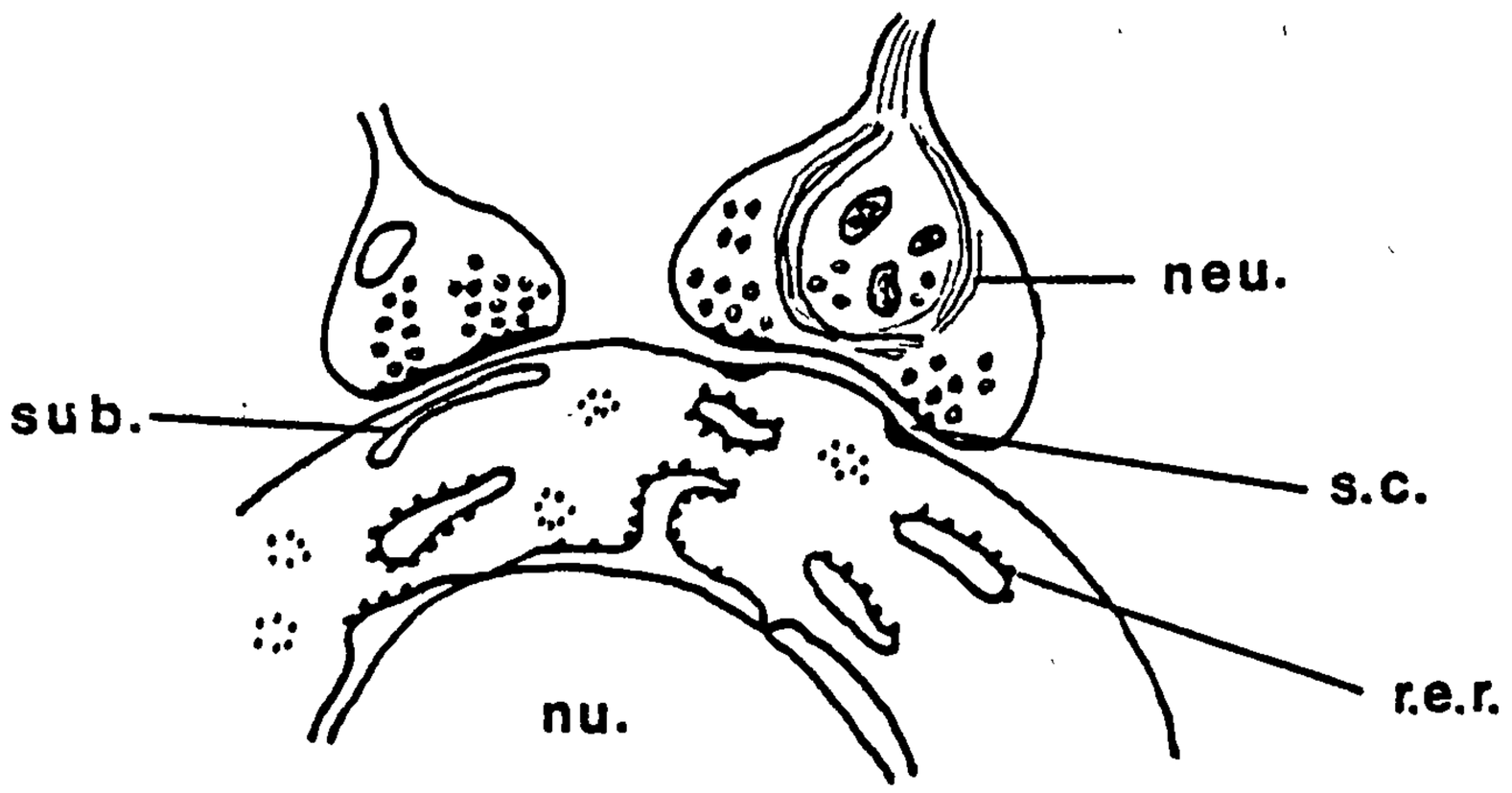
Fig. I.2. Diagrams to show the variety of structure found in chemically transmitting synapses (from Whittaker and Gray, 1962; Koelle, 1963)



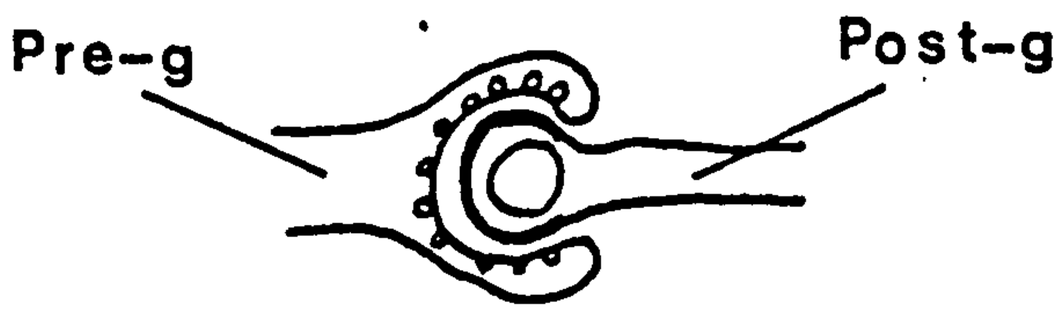

a) Axo-dendritic synapses




b) Synapse between an axon terminal and a dendritic spine




c) Two types of axo-somatic synapses




d) Synapse at autonomic ganglion

Fig. 1.2
Abbreviations: den., dendrite; tu., tubule; s.p., spine apparatus: sub., sub-synaptic cistern; nu., nucleus: r.e.r., rough endoplasmic reticulum; s.c., synaptic cleft (PAS positive material) + inter-synaptic filaments (?); neu, ring of neuro-filaments; Pre-g., Pre-ganglionic; Post-g., Post-ganglionic; s.v., synaptic vesicles; m.c., mitochon drion.

In an earlier work, Koelle (summarised by Koelle, 1968) investigated the various cat ganglia in an attempt to localise the AChE. He found that in the ciliary ganglion, which gives rise to cholinergic postganglionic fibres, all of the cells stained heavily at the cell membrane, throughout the cytoplasm, and along the length of the axons and dendrites as far as these could be traced. However, in the stellate ganglion despite high AChE activity in the entering preganglionic axons and their terminations on the ganglion cells, only very few ganglion cells stained for the enzyme. He proposed that these were probably the neurons which give rise to sympathetic cholinergic fibres to sweat glands and the vasodilator fibres. The majority of cells stained very faintly and probably represent the cells of origin of adrenergic fibres.

After further experiments conducted by Koelle (1968) using compounds of varying lipid solubility, two major features were found on the distribution of acetylcholinesterase in the ganglia:

Firstly, in the superior cervical and stellate ganglia of the cat essentially all of the functional AChE is presynaptic in contrast with its location at both pre- and post-synaptic membranes of the cat ciliary ganglion, and its predominantly postsynaptic distribution at the motor end plate of skeletal muscle.

Secondly, the presence of light to moderate concentrations of AChE in the ganglion cells give rise to adrenergic post-ganglionic fibres in practically all species examined, excepting the cat, as well as in the sensory, presumably non cholinergic, dorsal root ganglion cells of all species examined.

However, using an improved histochemical method (Koelle, et al 1974) the limitations of AChE to the presynaptic membrane has been questioned (Koelle, et al 1975). Electron microscopic studies of the cat superior cervical ganglion clearly showed that AChE was present at both the presynaptic and post-synaptic membranes, smooth endoplasmic reticulum and Schwann cell envelope. Koelle et al (1975), therefore think that the reason for disappearance of the ganglionic AChE consequent upon pre-ganglionic denervation is due to the loss of a trophic factor which maintains the AChE at the postsynaptic membrane as well as the atrophy of the presynaptic membranes themselves.

(iii) The Central nervous system. Sjöstrand (1938) first demonstrated that perfusion of the rat brain cortex with ACh caused a change in the electroencephalogram thus showing the probability of cholinergic mechanisms being involved in the central nervous system. The main motive behind much of the research on AChE in the central nervous system has been the identification of the cholinergic system present and correlation of the enzyme levels with those of cholineacetylase and acetylcholine. Burgen and Chipman (1951) measured the cholinesterase levels in the central nervous system of dog and found a very uneven distribution with a ratio of highest to lowest AChE activity (candate nucleus: subcortical white matter) of 400:1. This correlated well with the choline acetylase ratio for the same tissue (candate nucleus: dorsar spinal roots) of 540:1. Since these results, histochemical and iontophoretic techniques have corroborated and extended this aspect of the research. Cerebellum

is anomalous since, despite extremely high AChE levels, it has a very low cholineacetylase and acetylcholine content (reviewed by Silver, 1967).

Both light and electron microscopy have been responsible for the wealth of data on the localisation of AChE at the subcellular level but the latter technique is essential for identification of the really fine detail. This is exemplified by the work of Shute and Lewis (1966) who showed that in the rat hippocampus layers of AChE staining seen in the light microscope do not result, as one would expect, from concentrations of cholinergic endings but represent aggregations of cholinergic fibres forming the neuropil. This has been confirmed by Kokko et al (1969)

At supposedly cholinergic synapses (Fig. I.2) AChE is present in the pre and postsynaptic membranes (De Lorenzo, 1961; Torack and Barnett, 1962) although slight differences in the detailed amounts of staining at synapses are evident and this may be attributed to the methodology employed. Enzyme activity is not present in vesicles or mitochondria (Lewis and Shute, 1964).

Lewis et al (1965) demonstrated AChE in various cell types of rat brain and known cholinergic cells (e.g. ventral horns cells) showed intense staining within the sheets of endoplasmic reticulum. In contrast reputedly cholinoceptive cells (e.g. pyramidal cells of the hippocampus) possessed only slight activity localised in parts of the endoplasmic reticulum and nuclear envelope. Granular cells of the dentate gyrus did not stain at all and in the candate nucleus electron microscopy showed the enzyme to be mainly

localised to synapses (Lewis and Shute, 1964). As a general rule the results seem to indicate that fibres staining for AChE tend to form axodendritic rather than axosomatic synapses (Silver 1967).

Navaratnam and Lewis (1970) showed that in rat spinal cord neurons the AChE is principally localised within the cisternae of the endoplasmic reticulum and although the nucleus proper was unstained, activity was concentrated within the space between the membranes of the nuclear envelope. The plasma membrane of the cell body in general shows no enzymic activity but staining of the axonal membranes was frequently observed. Almost identical results have been obtained by Kokko et al (1969) with cerebral cortex.

A crucial point bearing on Nachmansohn's theory of conduction and transmission (to be discussed later) is the localisation of AChE in the excitable membrane of axons. In unmyelinated fibres evidence that it is localised in the membrane has been obtained (Schlaepfer and Torack, 1966). However, until quite recently the apparent lack of the enzyme in myelinated nerve fibres was a stumbling block for Nachmansohn but when Brzin (1966) applied Triton X-100 to slices of isolated sciatic nerve the enzyme was seen to be present in the plasma membrane. Further demonstration has since been obtained (Tennyson's work reported by Nachmansohn, 1971).

One further aspect of the localisation which may prove of importance, provided it is not a staining artifact, is the apparent localisation of the enzyme in patches rather than being equally distributed along the membrane. This has been demonstrated in muscle cells of plaice (Lundin et al, 1968) lobster nerve membranes (De Lorenzo et al, 1969) and squid giant axon (Brzin et al, 1965)

Much of the modern work is based upon studies involving investigation of nervous tissues grown in culture as these preparations have been shown to resemble in vivo preparations very closely (Bunge et al, 1965). In tissue cultures of spinal cord, large cells have been observed staining for AChE and these have been suggested to be motoneurons (Hösli et al, 1975). AChE was found in the cytoplasm of the cell body, on the outside of the soma and in the dendrites (Hösli and Hösli, 1971) but butyrylcholinesterase was localised in the glial cells. In cultures of cerebellum and brain stem a similar localisation was observed although here it was also found in the axon (Hösli and Hösli, 1970). AChE was also observed extracellularly although whether this was on the outside of the neurone or due to diffusion is questionable (Koelle, 1963).

Recently, regional studies have been made of AChE in the brain of pig (Knutsen et al, 1975) and in the central nervous system of the frog (Nistri et al, 1975). Both groups showed definite regional differences. In the pig brain, the caudate nucleus showed the highest activity of AChE and the cerebral cortex, the lowest. In the frog brain, the telencephalon showed the lowest concentration of enzyme and the rhomboencephalon the highest while the spinal cord had the highest levels of AChE in the cervical portion and lowest levels in the thoracic section.

In summary, it appears from histochemical evidence that AChE in the central nervous system is synthesised mainly within the cell bodies of the cholinergic cells themselves. In the soma the enzyme is localised within the lumen of the endoplasmic reticulum,

in the axon it is localized on the neuronal plasma membrane and at the terminal staining is very heavy and confined to the external membrane. This evidence suggests the enzyme is secreted onto the surface of the plasma membrane and flows down the outside of the neuron, accumulating at the terminal (Whittaker, 1969).

## B. ISOLATION

"The present work has been complicated by the fact that acetylcholinesterase is bound fairly tightly to membranes and this factor has had to be considered during the extraction and purification steps." (Nachmansohn, 1971).

### 1. Extraction

Methods for solubilizing AChE from the membrane have depended largely on the source of the enzyme. The tissues most commonly used have been: electric organ tissue (Nachmansohn and Lederer, 1939), erythrocyte stroma (Cohen and Warringa, 1953), and brain (Morton, 1950). The various techniques used reflect how different workers view the relationship of AChE with the membrane. Singer and Nicolson (1972) categorise membrane proteins as integral or peripheral. By their definition, AChE may be a peripheral protein because it can be extracted by the relatively mild procedure of high ionic strength media from erythrocyte ghosts (Mitchell and Hanahan, 1966) and electric tissue (Silman and Karlin, 1967), or the incubation of brain in ion-free media (Chan et al, 1972; Hollunger and Niklasson, 1973) or increasing the pH of incubation of brain tissue (Hayden et al, 1973). However Aloni and Livne (1974) point out that Singer and Nicolson's definition is equivocal because for example the AChE is only dissociable at high ionic strength from erythrocyte ghost but not from intact erythrocytes (Mitchell and Hanahan, 1966). Also various other workers have had to resort to using more severe techniques for removing the enzyme from the

membrane, notably detergents. Paniker et al (1973) studied the efficacy of several solubilizing agents such as detergents, chelating agents and salt, and they came to the conclusion that AChE is an integral protein of the erythrocyte membrane because removal of the enzyme was not selective but followed the solubilization of other membrane proteins. Jackson and Aprison (1966) and Ho and Ellman (1969) originally used detergents to solubilize the enzyme and they found an almost quantitative shift of activity from the particulate fractions into the 100,000 g. supernatants. Since then, the use of non-ionic detergents, particularly Triton X-100 (an alkylphenylpolyoxyethylene condensate) to extract AChE has become almost routine due to their great efficiency (Wright and Plummer, 1972; Bellanger et al, 1973; McIntosh and Plummer, 1973; Leterrier et al, 1974; Plummer et al, 1975; Vigny et al, 1976; Reavill and Plummer, 1978).

It has been found that many membrane enzymes tend to be denatured by certain detergents particularly those that are ionic such as sodium dodecyl sulphate, and to a lesser extent, the bile salts such as sodium deoxycholate (Coleman, 1973). The most successful ones have been the non-ionic detergents such as Brij, Triton and Lubrol series. Helenius and Simon (1975) suggested that this is due to their efficiency in dispersing the membrane lipid.

It has been shown that the physico-chemical properties of some of the molecular forms of AChE from Torpedo Californica are modified in the presence of detergent (Taylor et al, 1975). Evidence of such modifications has been reported by Ott et al

(1975, 1978) working on human erythrocyte membrane enzyme where in presence of Triton X-100 only one peak of activity was detected by sucrose gradient centrifugationand after removal of detergent up to five bands were detected. They suggest that Triton brings about disaggregation of the multiple forms which reaggregate upon removal of the detergent. Such reaggregation was prevented by.the use of chaotropic agents indicating that hydrophobic interactions are very important in the formation of oligomeric forms. According to Hatefi and Hanstein (1974), chaotropic ions are thought to decrease hydrophobic interactions by disturbing the water structure and increasing the solubility of lipophilic compounds in water. However, Vigny (1978) showed that the use of detergents has no effect in the catalytic activity so that it appears that the tertiary structure of the acetylcholinesterase subunit contains a catalytic domain which is relatively independent of the detergent, membrane and inter-subunits binding domaine.

In order to avoid possible interferences due to the use of detergents on the molecular forms of acetylcholinesterase attempts have been made to solubilize the enzyme in media not containing detergents. Hollunger (1973) brought up to 90% of the total AChE present in homogenates from calf brain into solution by incubating the particulate fraction in an ion-free media. Adamson (1977) has used the Hollunger method to solubilize the enzyme from three different tissues of mouse: brain, erythrocytes and muscle and he found that the enzyme from erythrocyte and muscle is much more tightly bound to the membrane than in the case of brain; 80% of the

brain enzyme was solubilized against 40% for muscle and erythrocyte.

The electric organs of various electric fish are somewhat anomalous in that they have very high levels of AChE and AChR (acetylcholine-receptor) and very little of any other proteins. This fact has made the electric fish an obvious candidate as a source of the enzyme. The actual electroplax has been shown to be phylogenetically derived from muscle (Nachmansohn, 1959). In Electrophorus electricus various conditions for extraction have been tested, revealing that most of the native enzyme requires high ionic strength for its extraction (Silman and Karlin, 1967; Massoulié and Rieger, 1969; Dudai and Silman, 1974 a,b)

It seems quite clear nowadays that the enzyme from mammalian sources is more tightly bound to the membrane than in the case of fish but it would be fruitless to compare this organ with preparation of mammalian nervous tissue as it is so specialized in its action.

## 2. Purification

Early purification procedures could only be applied originally to the crude enzyme obtained from Electric eel organ because this was the only source known to contain acetylcholinesterase in quantities large enough to obtain a reasonable yield of enzyme after all the purification steps.

Rothenberg and Nachmansohn (1947) introduced the use of ammonium sulphate fractionation of the crude enzyme obtained from the Electrophorus electricus electroplax. This procedure was

modified by Lawler (1959) who obtained a preparation with a recovery over the original crude enzyme of 15%. Kremzner and Wilson (1963) improved the technique by homogenizing the electric organ in 5% ammonium sulphate and then sequentially chromatographing the enzyme through benzyldiethylaminoethyl cellulose, Sephadex G-200. Cellex-P and diethylaminoethyl cellulose. This procedure gave a yield of 9% with an activity of 660 units/mg protein and a purification of about 370 times. Leuzinger and Baker (1967) using the same technique obtained crystals of the enzyme with an activity of 750 units/mg protein and this preparation was used in the determination of the crystal structure of a globular form (Leuzinger et al, 1968; Chotia and Leuzinger, 1975).

Owing to the lengthy procedures involved in the established purification several groups have developed affinity chromatography methods. In affinity chromatography (Cratrecasas and Anfinsen, 1971) a reversible specific inhibitor of an enzyme is covalently bound to a resin. The inhibitor-resin conjugate will then specifically adsorb the enzyme from a mixture of proteins, and the enzyme can subsequently be eluted in purified form, preferably by the use of a soluble inhibitor.

Kalderon and co-workers (Kalderon et al, 1970; Dudai et al, 1972) developed a method for the purification of the 11S form of AChE obtained either by controlled tryptic digestion or prolonged autolysis of electric organ tissue. They employed the AChE inhibitor [N - (ε-aminocaproyl)-p-amino-phenyl] trimethyl-ammonium bromide hydrobromide (Table I-2) covalently linked,

via its $\varepsilon$-amino group, to CNBr-activated sepharose by the method of Axen et al (1967). AChE containing solutions were applied to the affinity column and purified enzyme subsequently eluted with decamethonium bromide. These workers claimed comparable purity to that of Leuzinger (1971).

More recently the Israeli group (Dudai et al, 1972; 1973) have developed a column for the direct purification of the native molecular forms of the enzyme (those present in fresh electric organ tissue before tolnene treatment or tryptic digestion).

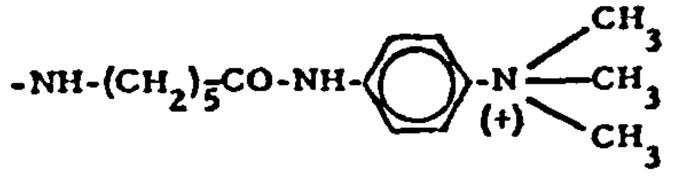
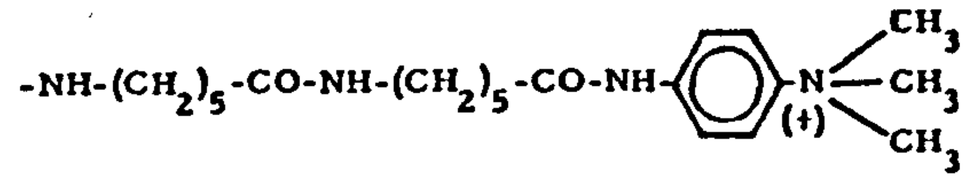
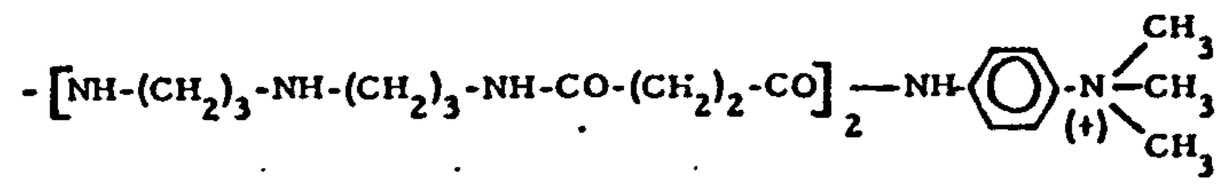
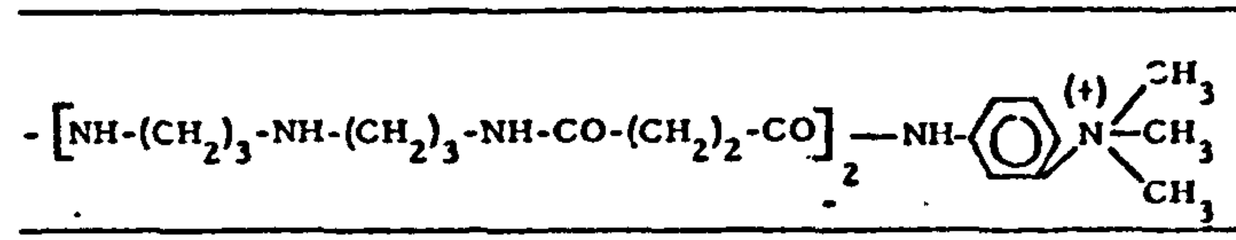
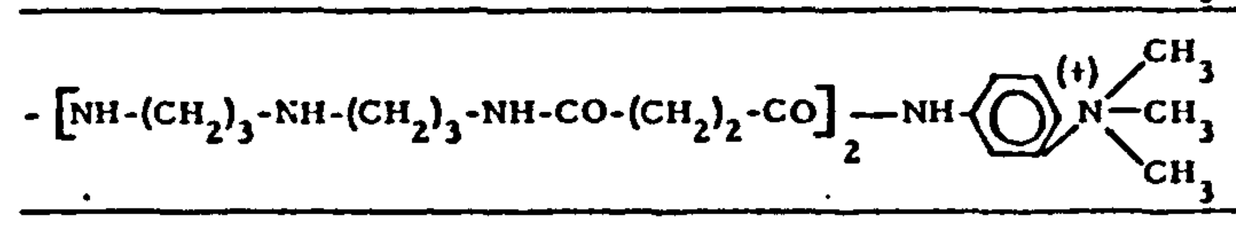
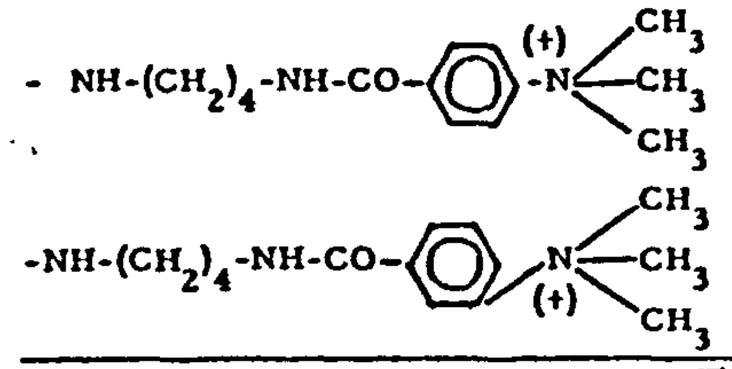
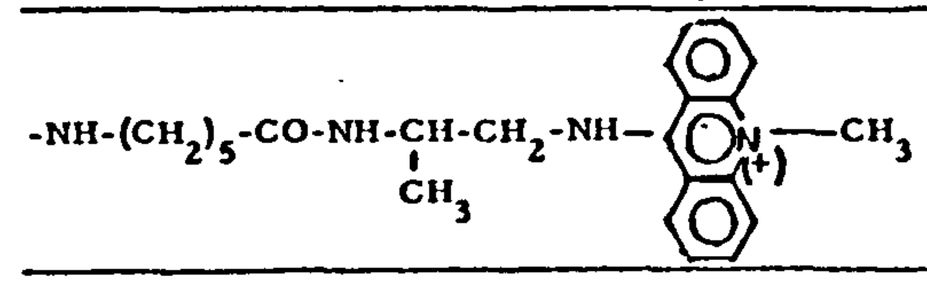
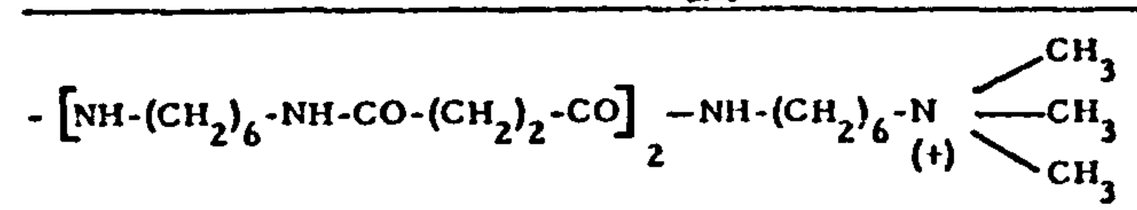
Several other groups have used the phenyltrimethylammonium (PTA) ligand when attached to various spacer arms as the AChE inhibitors. Berman and Young (1971), purified the eel AChE using the meta and para derivatives of this ligand but only the meta derivative was successful in retarding the erythrocyte enzyme. Similarly, different workers using enzyme from mammalian tissues have showed greater retention of AChE on columns containing the meta-PTA ligand rather than para-PTA ligand (Chan et al, 1972; Yamamura et al, 1973; and Dawson and Crone, 1974).

Dawson and Crone (1974) also compared elutions when Triton X-100 was present with elutions when the detergent was absent. This is of particular interest when purifying Triton solubilized enzyme. They showed that an additional peak of activity was eluted when the detergent was incorporated into the elution media. This agrees with Chrone's (1971) data in which he found that some Triton solubilized enzyme adsorbed to an agarose column if the detergent was not present during elution. However, it is not consistent with the work of Yamamura et al, (1973) who managed to purify Triton

solublized guinea pig brain AChE without including Triton in the elution buffer. Dawson and Crone (1974) explain this by saying that the detergent present with the applied enzyme maintained high enough levels of detergent throughout all stages of the procedure. Goodkin and Howard (1974) purified the enzyme from rat brain in the presence of Triton X-100 and when the detergent was omitted the recovery was markedly reduced.

The most important affinity chromatography systemswhich have been described for acetylcholinesterase are listed in the Table I.2.

TABLE I.2

| Structure of Spacer Arm and Ligand | Enzyme | Wash Buffer | Elution Medium | References |
|---|---|---|---|---|
|  | Electrophorus trypsin-treated lytic form (G) | 0.1M NaCl<br>0.01M P-P<br>pH7 | 0.01M Decamethonium<br>0.1M NaCl<br>0.01M P-P pH7 | 1. Kalderon et al (1970)<br>2. Dudai et al (1972a)<br>3. Dudai et al (1972b) |
|  | Electrophorus autolyzed Lytic form (G) | 0.02M P<br>pH 6.9 | Salt gradient<br>0.1M-0.15M NaCl<br>or KCl | 4. Rosemberry et al (1972)<br>5. Chen et al (1974) |
|  | Electrophorus | 0.1M NaCl<br>0.04M $MgCl_2$<br>pH 7.8 ($CO_3HNa$) | 0.01M-Tensilon<br>0.1M-NaCl<br>0.04M $MgCl_2$<br>pH 7.8 ($NaCO_3H$) | 6. Berman et al (1971) |
|  | Bovine Erythrocyte | 0.01M P-P<br>pH 7.5 | 0.01M-Tensilon<br>0.1M NaCl<br>0.04M P-P 7.5 | 7. Berman et al (1973) |
|  | Bovine brain solubilized without deter gent | 0.1M NaCl<br>0.03M P-P<br>pH8 | 0.01M Tensilon<br>0.1M NaCl<br>0.03M P-P pH8 | 8. Chan et al (1972) |
|  | Guinea pig brain; extracts contained 0.7% Triton X-100 | 0.5M NaCl<br>0.01M P-P<br>pH 7.4 or<br>0 2M NaCl<br>0.01M P-P<br>pH 7.4 | salt or choline chloride gradient<br>( 0 4M NaCl)<br>0-0.5M choline chloride gradient | 9. Yamamura et al (1973) |
|  | Electrophorus asymmetric forms. Pig brain extracts contained 1% Triton X-100 | 1M NaCl<br>0.01M P-P pH7<br>P-P pH7<br>0 03M | 0.02M Decamethonium<br>1M NaCl, 0.01M P-P pH7<br>0.01M Decamethonium<br>0.03M P-P pH7 | 10 Christian et al (1972)<br>11. Dudai et al (1972)<br>12. Reavill et al (1978) |
|  | Torpedo | 0.5M NaCl<br>0.02M P-P<br>pH7 | 0.2M tetramethylammonium bromide<br>0.5M NaCl.<br>0.02M P-P pH7.0 | 13. Hopff et al (1973) |

## C. STRUCTURE

The IUPAC-IUB (1971) defined the term 'multiple forms of the enzyme' as a broad term covering proteins possessing the same enzyme activity and occurring naturally in a single species. This description applies to AChE rather than the term isoenzyme which, strictly speaking, applies to multiple forms of the enzyme arising from genetically determined differences in primary structure and not to those derived by modification of the same primary sequence. Thus electric organs acetylcholinesterase multiple forms cannot be correctly called isoenzymes, since they rather represent different stages of disruption of an original complex structure and apparently are built up of one main type of subunit which may be secondarily cleaved into smaller polypeptides.

It may be that in some cases acetylcholinesterase multiplicity could arise from the existence of real isoenzymes. One of the best indications for this seems to be the occurrence of two chromatographically separable and electrophoretically different forms obtained by Shafai and Cortner (1971 a, b) from human erythrocytes. These two forms can be arranged under certain conditions and this process could be interpreted as:

$$\alpha 2 + \beta 2 \rightleftharpoons 2\alpha\beta$$

In this model, $\alpha$ and $\beta$ might be isoenzymes in the "strict sense". However, no similar situation has been found for preparations from other tissues and therefore is more desirable the use of multiple forms term than isoenzymes for acetylcholinesterase.

## 1. Molecular Structure of the Electric Eel Enzyme

Efforts at determining the molecular weight and subunit composition of AChE have mainly been directed at the enzyme present in electric organ. Studies on partly or highly purified preparations using polyacrylamide gel electrophoresis or density gradient centrifugation have shown that AChE exists in several multiple forms of varying molecules weights.

Rothemberg and Nachmanson (1947) were the first workers to suggest that AChE existed in several forms from the nature of the sedimentation coefficients. Lawler (1963) indicated the presence of AChE molecules ranging in molecular weight from $3.5 \times 10^5$ to $3.1 \times 10^7$ and consisting of multiple forms of the same subunit and also stressed that a polymer with a molecular weight of about 30 millions could not be present in the membrane as a sphere but might exist as a flexible rod.

Since these early findings several laboratories have shown conclusively that AChE, in solution, exists in different multiple forms (Hargreaves et al, 1963; Massoulié and Rieger, 1969; Dudai et al, 1972a; Massoulié et al, 1975; Silman and Dudai, 1975; Cartaud et al, 1975; Bon et al, 1976; Johnson et al, 1977). There seems to be fair agreement, especially between Massoulié's group and Dudai's group as to the subunit composition of the enzyme from electric organ. The predominating species (by sedimentation studies) are -18S (1,100,000), -14S (780,000) and-9S (430,000). These three forms behave as highly asymmetrical particles, all of them aggregate at low ionic strength and are sensitive to ultra-

sonic vibrations, which degrade them into more globular, but still active molecules. Two main forms are globular with sedimentation coefficients of 11.8S (370,000) and 7.7S (180,000) and do not aggregate at low ionic strength. (Massoulié et al, 1971; Rieger et al, 1972; Rieger et al, 1973a). Similar findings have been obtained by Grafius and Millar (1965); Dudai et al (1972a); Dudai et al (1973). The asymmetrical molecules can be degraded by proteolysis. Another globular molecule has been detected with a sedimentation coefficient of 11.1 S, quite distinct from that of 11.8 S. All of them presented the same Km value and inhibition by excess of substrate. There is no conversion from one form to another by exposure to high temperatures. According to electron microscopy (Rieger et al, 1973) the 11.8S and 7.7S forms are composed of four and two globules (the diameter of which is about 6.7 nm). On the other hand, the -18S, -14S and 9S forms are built of two parts: a globular "head" made up of globules similar to those found in the 11.8S and 7.7S forms and a rod-like tail. This element was about 50 nm long and 2.3 nm thick.

In a superb microscopic study Cartaud et al (1975) reported that the asymmetric forms are composed of an assembly of tetrameric units. The total number of subunits in the 18S form is 12, displaying a 40 nm tail. Occasionally the three tetrameric assemblies forming the head piece are found quite apart from each other. Nevertheless they appear still connected by filaments probably originated from the splitting or degradation of the tail structure. The tail piece of some of the polymeric molecules appears to be split into

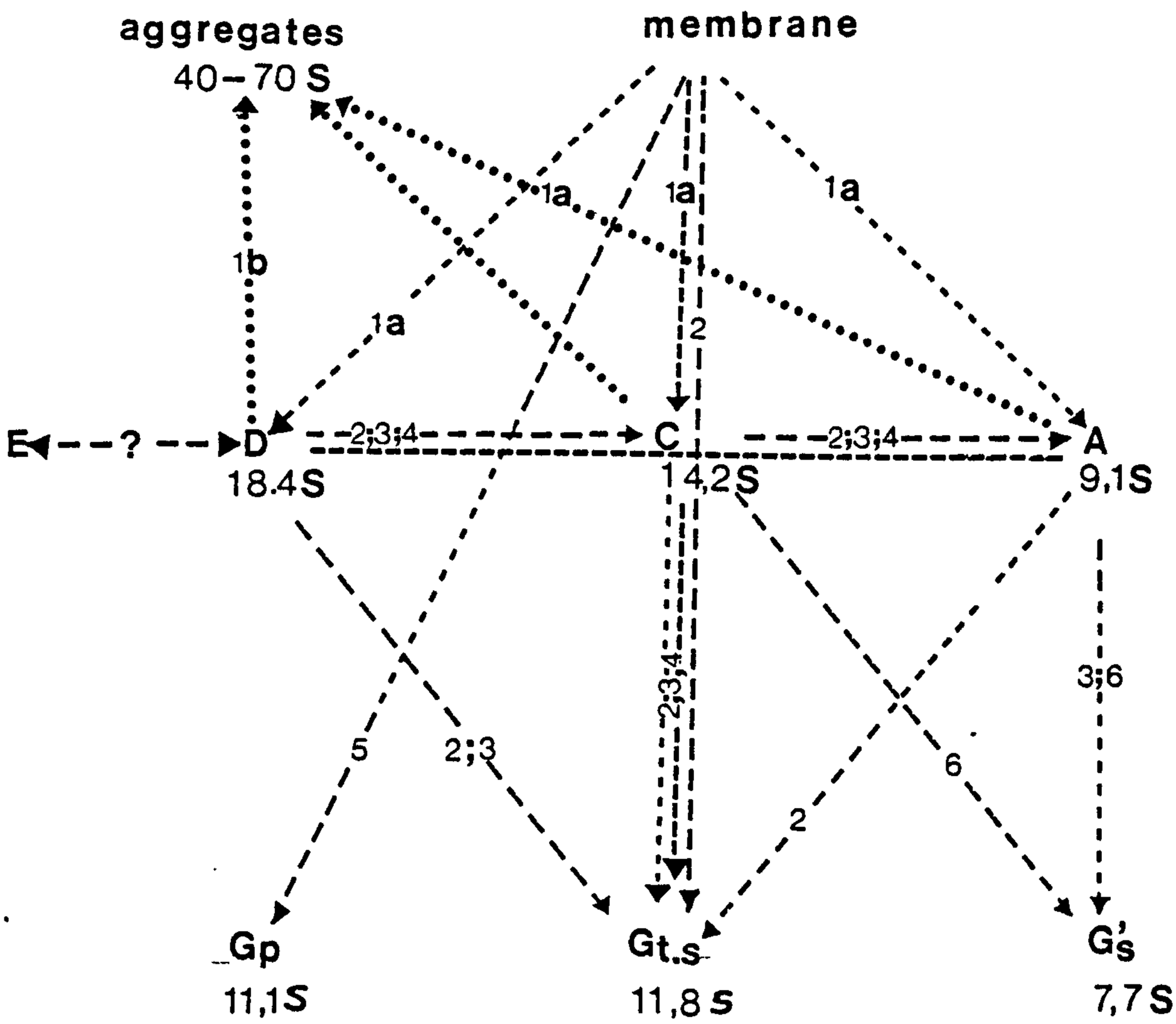
3 individual filaments each associated with one tetramer. The filaments seem to be intertwined in the distal half of the strand and clearly separated in the proximal half. They suggested that the tail is formed by association of three α-helices or organised in a collagen like 3 stranded helical conformation. From calculation of the size of tail and comparing with a polypeptide chain dimension they calculated an average molecular weight for each strand of 40,000 and for the total tail - 120,000. They supposed that the conversion from 18S to 14S and from 14S to 9S might involve the loss of one tetrameric complex and an additional molecular mass ranging from 60,000 to 70,000.

The 11S preparation is a globular protein (Kremzner and Wilson, 1964), and it has been shown to be a tetramer with four similar active-site-bearing subunits each of molecular weight 80,000, giving an overall molecular weight of 300,000-350,000. This 11S form has not been found in fresh electric organ tissue. However all the asymmetric forms áre converted by proteolysis or autolysis to the 11S form. Wermuth, Ott et al, have summarized the interconversions diagrammatically (see Fig. I.3).

The subunit molecular weight has caused much argument and the actual value has still not been conclusively resolved. Leuzinger et al (1968) after centrifuging AChE in guanidine and subjecting it to SDS-gel electrophoresis, concluded that the enzyme was a tetramer (molecular weight 240,000) consisting of two different subunits of molecular weights 64,000, thus giving a dimeric hybrid $(\alpha\beta)_2$. However, Dudai et al (1972), by SDS-polyacrylamide gel electrophoresis in the presence of reducing agents, showed

FIG. I.3

Ways of Solubilization of AChE from the Membrane and the Interconversions among the Various Forms




1a. High ionic strength; 1b. low ionic strength; $Ca^{+2}$; 2. trypsin; 3. sonication; 4. spontaneous; 5. autolysis; 6. triton X-100.

that the elongated forms of AChE contain the same 80,000 polypeptide observed in 11S AChE, with small amounts of 60,000 and 20,000 components produced from the 80,000 polypeptide by autolysis (Dudai and Silman, 1974a; Rosemberry et al, 1974). These results agree fairly well with those of Powell et al who referred to the subunits as light (l) and heavy (h) and who denote the tail as 'q'. Using this explanation, it is possible to obtain a whole range of molecular weights by using different permutations of the different subunits. Wermuth et al (1975) have summarised the different combinations of subunits (Table I.3). Rosemberry et al (1974) disagree with the dimeric hybrid $(\alpha\beta)_2$ model and propose that the tetramer is composed of identical subunits as a dimer of dimers $(\alpha_2)_2$. In their results they show that the subunit may be cleaved into 2 polypeptides, one of which may be further cleaved into two more polypeptides (Fig I.4). They suggest that the dimeric hybrid $(\alpha\beta)_2$ model has been based on observations of the subunits at various stages of proteolytic cleavage. More information about the structure of the electric eel enzyme has been found by SDS-polyacrylamide gel electrophoresis, in the absence of reducing agents. Whereas 11S AChE, under such conditions, contains primarily the 160,000 dimer, electrophoresis of 14S + 18S without reduction reveals the presence of two species with molecular weights ~360,000 and ~460,000. Electrophoresis of separated samples of 14S and 18S showed that the 360,000 component is derived exclusively from 14S and the 460,000 from 18S (Silman et al, 1978). Despite the higher concentration of hydroxyproline, hydroxylysine, glycine in the 14S + 18S preparation than in

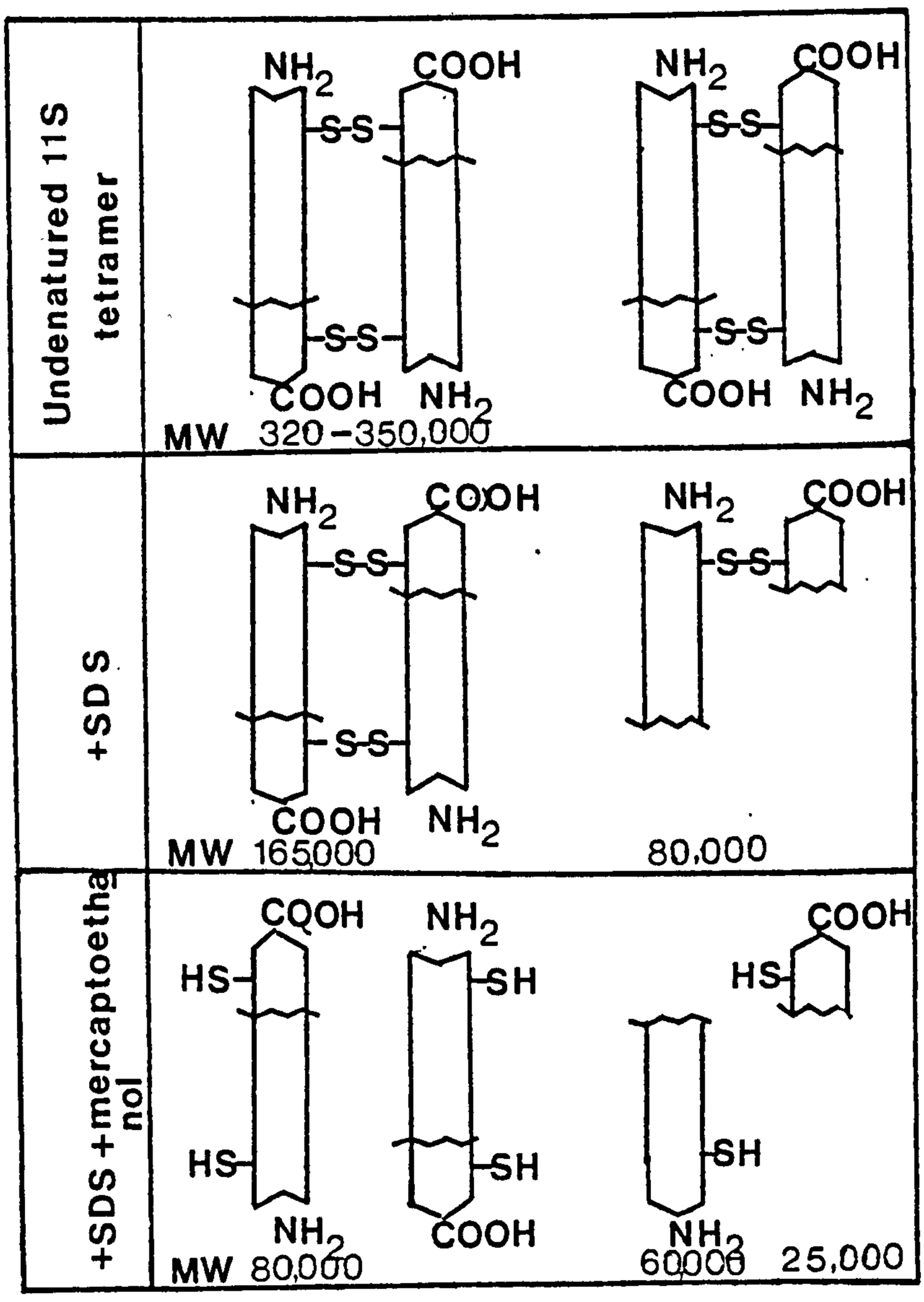
TABLE I.3

Properties of Elongated and Globular Forms of AChE

| Form | Subunit composition | Molecular Weight (daltons) | Specific activity (U/mg) | Stoke's radius (nm) | Sedimentation coefficient (S) | Occurrence in E.M. |
|---|---|---|---|---|---|---|
| Elongated | | | | | | |
| A | $h_2I_2q$ | 430,000 | 14,300 | 12.4 | 8.5-9.1 | grape-like |
| C | $h_4I_4q$ | 780,000 | | 14.4 | 14.2 | grape-like |
| D | $h_6I_6q$ | 1,100,000 | 13,300 | 15.0 | 18.4 | grape-like |
| E | $h_8I_8q$ | 1,560,000 | | 17.0 | - | dumbbell |
| Globular | | | | | | |
| Gp | $h_2I_2$ | 260,000 | 15,200 | 8.2 | 11.1 | tetramer |
| Gt | $h_2I_2$ | 290,000 | - | - | 11.8 | tetramer |
| G's | hI | 155,000 | - | 6.4 | 7.7 | dimer |

FIG. I.4

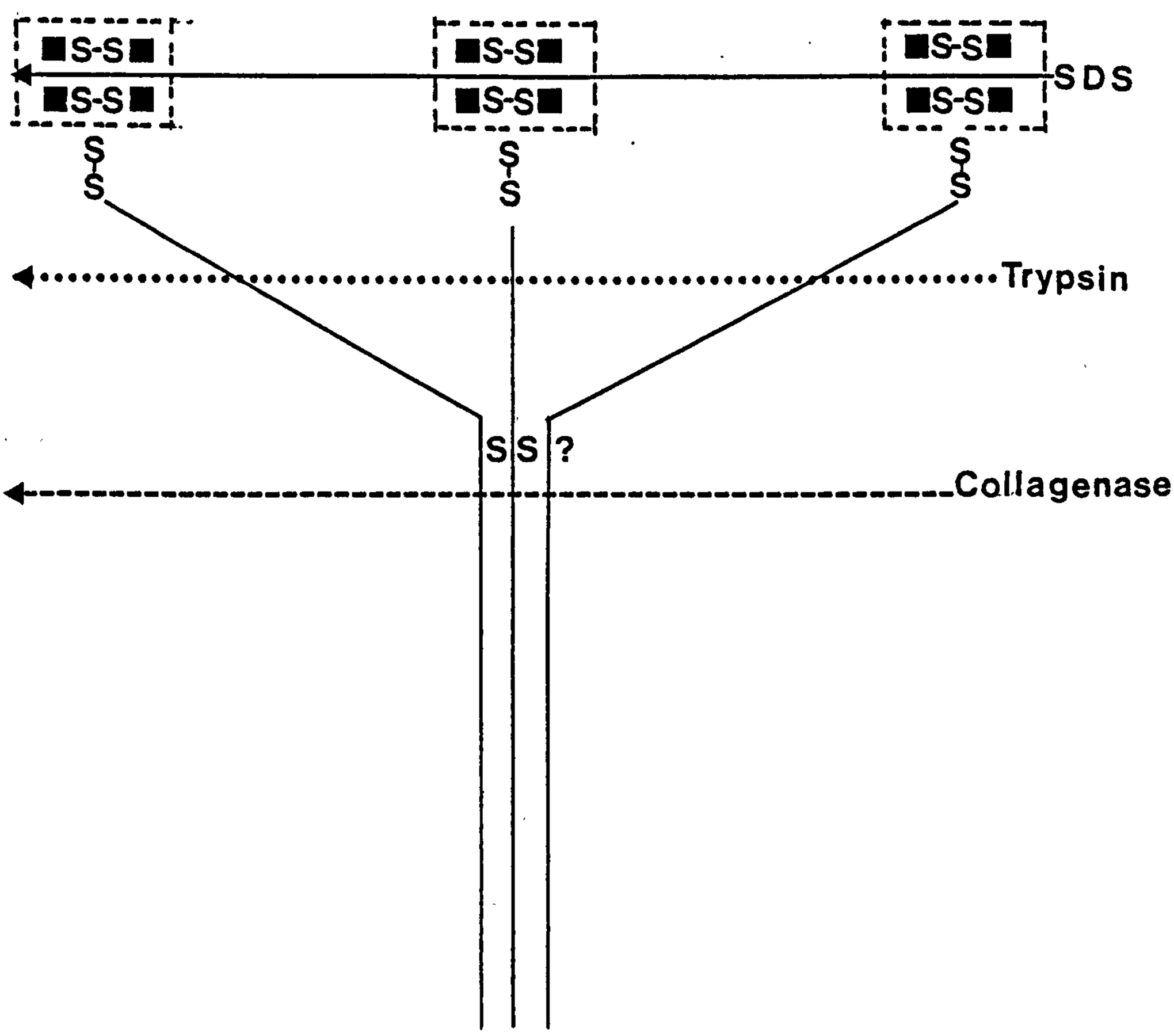
Schematic model of subunit structure of 11S AChE together with the products to be expected under the conditions employed for SDS-polyacrylamide gel electrophoresis, both in the presence and absence of β-mercaptoethanol subsequent to cleavage of either one or both of the polypeptides of the dimer. The site where cleavage can occur is indicated by a serrated line.



the 11S. This finding agrees with the predicted values if the tail was a triple helix of collagen as is seen by the electron microscope. In the same paper Silman has observed the effect of collagenase and trypsin on purified AChE. Collagenase, like trypsin, abolishes the capacity of AChE to aggregate at low ionic strength. However, where-as trypsin converts the elongated form to 11S form, collagenase converts the 18S and 14S AChE to non-aggregating species with significantly higher sedimentation coefficient 20S and 16S respectively. SDS-polyacrylamide gel electrophoresis without reduction also showed that collagenase acts differently from trypsin. Whereas trypsin completely converts the heavy components in 14S + 18S AChE to the 160,000 dimer found in the 11S AChE, collagenase produces two new components with molecular weights of about 290,000 and 185,000. This process is retarded by the collagenase inhibitor o-phenanthroline. Finally, Silman proposes a model to explain the effects of SDS, trypsin and collagenase on elongated forms of AChE (Fig. I.5). The 80,000 subunits are linked by disulfides bridges to form 160,000 dimers which associate in pairs to form tetramers. In the 18S form of AChE, each tetramer is attached, presumably by disulfide bonds to one strand of a collagen triple helix. SDS detaches only those dimers which are not covalently linked to the tail. Trypsin cleaves the tail close to the point of attachment to the catalytic subunits releasing intact tetramers. Collagenase cleaves at a point within the stem of the tail, removing that part of the tail responsible for aggregation at low ionic strength, but leaving intact a region which holds the tetra-mers together. The observed increase in sedimentation coefficient

FIG. I-5

Scheme showing suggested model of cleavage of 18S AChE by sodium dodecyl sulphate, trypsin and collagenase (After Silman I, Anglister L. and Gazit H. 1978)



may be attributed to the formation, by removal of the tail, of a more symmetric molecular structure, without a significant decrease in molecular weight, since the whole tail probably accounts for less than 10% of the total molecular weight of 18S AChE.

## 2. Composition of Acetylcholinesterase from Other Sources

Some studies have also been carried out on multiple forms of AChE from other sources such as brain, diaphragm and erythrocytes, although results have not proved to be so fruitful as the electric tissue enzyme. Hollinger and Niklasson (1973) isolated AChE from bovine brain with a molecular weight of 80,000 which aggregated on storage. If the enzyme was prepared in the presence of DEAE-Sephadex A-25 it no longer aggregated suggesting the removal of an aggregating factor. Recently Bon et al (1978) have discovered that the low ionic strength aggregation of Electrophorus asymmetric forms depends upon an aggregating agent, present in large excess in the electric organ extracts. The aggregate appeared in electron micrographs, as bundles of a few molecules, the tail of which was linked in a side-to-side association, with the head groups at both ends. The properties of the aggregating factor suggested that it might be a non-protein polymer interacting with positively charged groups of the enzyme tail. They found that in such aggregating preparations, the chondroitin sulfate content fully accounts for its aggregating capacity. This agrees well with its sensitivity to specific-mucopolysaccharidases. They suggest that in the synaptic cleft AChE may be immobilised through high-affinity, ionic strength-dependent interactions of its

tail moiety with polyanions, with the tail bound to glycosaminoglycans of the chondroitin sulfate type. They do not discard the possibility that other negatively charged groups such as phosphates of acidic membrane phospholipids might also play an important role.

McIntosh and Plummer (1973) detected between two and six forms of pig brain AChE, the most frequently found being 60,000, 130,000, 198,000, 266,000 and 350,000. They also found a sedimentation coefficient of 11-12S for Triton solubilized enzyme and -11S, -15S and -19S for naturally soluble enzyme (Plummer et al, 1975). Wenthold et al (1974) using a preparation from rat brain found four main forms by gel filtration with molecular weights of about 150,000, 320,000, 500,000 and 650,000 where the lightest account for 60% of the total activity. By gel electrophoresis in presence of SDS, they found that each of the six bands separated by isoelectro-focusing (pI 5.51, 5.42, 5.30, 5.21, 5.10 and 5.04) yielded a single band with MW of 80,000. They also showed that the six bands presented glycoprotein nature. Rieger and Vigny (1976) have found two forms of AChE from rat brain. They show that one of them is easy to solubilize without detergent (4.6S) and the other (10S) hard to solubilize in absence of detergent. The lighter form has a molecular weight of about 115,000 and the heavy one 435,000. In rat diaphragm muscle, Hall (1973) described three molecular forms of AChE with sedimentation coefficient of 4S, 10S and 16S, the last one being found only in the endplate region. Vigny et al (1976) have solubilized three active molecular forms of AChE from rat muscle and also they have detected the presence of one of these forms

(16S) only at the motor end-plate region of several skeletal muscles and because that form was never detected in smooth muscle extracts and for other reasons they suggest that the 16S form could be an excellent marker of the neuro-muscular junction. In a later paper, Rieger et al (1976b) found molecular forms with the same solubilization and sedimentation properties in several tissues or cells from rat or mouse: brain, erythrocytes, glial or neuroblastoma cell and in all cases the molecular forms found are the same but with different relative proportion. They have found the 16S form in superior cervical ganglia from mouse but not in neuroblastoma cells. Another interesting comparative study has been carried out by Adamson (1977) on the properties of the enzyme from mouse brain, erythrocytes and muscle in order to see if there were any immunological differences between them. All the preparations showed to contain a single active small molecular weight component of 80,000-82,000 which produced higher molecular weight forms by aggregation. The partially purified enzyme from brain was used to prepare antibodies and the immunoglobulin seemed to react with all three of the enzymes.

Acetylcholinesterase of brain, ciliary ganglia, nerves and muscles (normal and denervated) of young chicken has been investigated for Vigny's team (1976). Four forms, according to their sedimentation coefficients were identified. (a) A low molecular weight form of 4S, which seems to be detectable mostly in peripheral nerves; (b) Two intermediate forms, 6.5S and 11S, which seems to be ubiquitous; (c) A high molecular weight form, 19.5S

which is detectable in ganglia and normal muscle and disappears from denervated muscles. None of these forms were considered to be artefacts of the Triton X-100 extraction, since they were equally detectable in a detergent free media.

In adult chicken skeletal muscle three forms of AChE have been observed, a major 6.5S and two minor 11S and 19.5S components (Vigny et al, 1976). Both in cultures of embryonic pectoral muscle and in homogenates of breast muscle from 18-day embryos, only one major species of AChE was found with a sedimentation coefficient of 6.5S, but if the tissue was frozen at least once a new peak of 13S was detected. The same effect was observed after autolysis or tryptic digestion. From the Stokes radius it seems that the 6.5S molecule is quite asymmetric and the one with 13S is more 'globular'. The proposed molecular weights for these forms are 260,000 for the 6.5S and 366,000 for 12.5S.

Ott et al (1975) obtained a molecular weight value of 80,000 for the AChE subunit from the detergent solubilized human erythrocyte. They showed one peak of 6.3S by sucrose gradient centrifugation in the presence of Triton X-100 and by isoelectric-focusing five molecular forms with isoelectric points of 4.55, 4.68, 4.81, 4.98 and 5.18. After removal of the Triton X-100, five molecular forms were detected by sucrosegradient centrifugation with coefficients of 6.3S, 10.2S, 12.2S, 14.2S and 16.3S. They suggest that human erythrocyte AChE, solubilized by Triton exists in various form differing in net charge but of apparently similar molecular dimensions. In a more recent paper, Ott and Brodbeck (1978) have found up to eight molecular forms of AChE by sucrose gradient

centrifugation in a zonal rotor. Hydrodynamic properties of the four most abundant oligomers were investigated by analytical ultracentrifugation. The $S_{20,w}^{-}$ values obtained from these components ranged from 12.5S to 19.0S with corresponding molecular weights between 500,000 and 1,131,000. These oligomers are built up of 6-14 subunits. Analysis of each isolated homogenous form after storage for 6 months at 4°C revealed interconversion into the original eight components. Upon addition of Triton X-100, the oligomers disaggregate to yield a single 7S form, which reaggregated upon removal of the detergent. Such aggregation could be prevented by chaotropic ions indicating that hydrophobic interactions are important in the formation of these oligomeric forms.

The above review of results illustrates the varying findings published by different laboratories. However, there does seem to be some correlation in the results suggesting a basic MW species approximately of 60,000-85,000.

Whether the enzyme exists in the membrane as a monomer or a multiple molecular aggregate has not been definitively elucidated. With the existence of a collagen-like tail projecting from a bunch of several subunits, it looks quite possible that the enzyme exists as a molecular aggregate being rooted on the membrane via the tail. However, Levinson et al (1974) found that the enzyme in the membrane is a monomer of molecular weight 75,000 and they suggest that the multiple forms of the enzyme observed in solubilised preparations are probably aggregates of this monomer.

## 3. Equivalent weight

The equivalent weight of a protein can be defined as its grams per mole of active sites. It is possible to determine this value if the protein is very pure provided that a suitable titrating agent can be found specific for the active site.

Many AChE titrants used have functioned by acylating the serine in the active site. Michel and Krop (1951) used radioactive $DF^{32}P$ to determine the normality of the enzyme whereas Kremzner and Wilson (1964) followed the loss in activity concommitant with phosphorylation by N,N-Dimethyl-S-(diethyl-phosphoryl) triethanolamine. Recently Vigny et al (1978) have used O-ethyl-$S^2$-diisopropylaminoethyl methyl-phosphonothionate to test the active site catalytic efficiency of molecular forms of Electrophorus, torpedo, rat and chicken. Another technique is to bind fluorescent ligands which exhibit diminished quantum yields when associated with the enzyme (Rosemberry et al, 1971; Mooser et al, 1972) or which are totally quenched when bound (Mooser et al, 1972). Taylor and Lappi (1975) used the ligand propidinium. which binds to a locus peripheral to the catalytic site and showed a ten-fold increase in fluorescence when bound. Rosemberry (1975b) arrived at a value for the equivalent weight of AChE of 76,000 g/mole of active sites which is close to the subunit molecular weight of eel 11S enzyme. The number of active sites per tetrameric molecule is thus the MW divided by equivalent weight which is the number of subunits per molecule. The value determined by several workers is four active sites per tetrameric molecule. (Mooser et al, 1972; Chen et al, 1974).

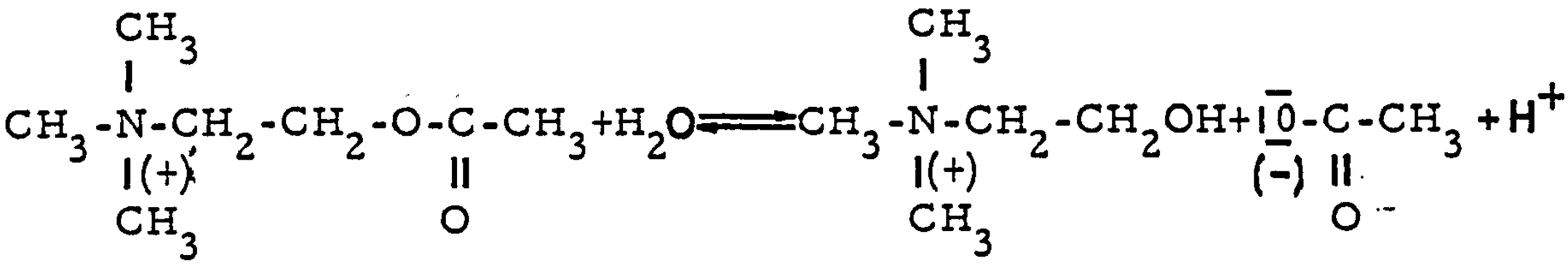
Recently Vigny et al (1978) have found that all the multiple forms of Electrophorus AChE possess identical turnover number per active site; therefore quaternary interactions among the catalytic subunits themselves and with the tail, do not interfere with the catalytic efficiency of the active sites. However, it is quite possible that some modified molecular forms might differ in their specific activity or even in Km values. This was true for torpedo, rat and chicken and also for the 10S form of rat in its membrane-bound state as well as that solubilized in the presence or absence of detergent.

They suggest that this behaviour may be generalized to other tissues. It looks also that the tertiary structure of the acetylcholinesterase subunit contains a catalytic domain which is relatively independent of the detergent, membrane and intersubunits binding domains. They consider that the size of the catalytic polypeptide, which seems to be in the range of 60,000-80,000 is in fact much larger than appears necessary for the existence of the catalytic site, since according to Hogberg-Raibaud and Goldberg (1977) the elementary globular organized unit seems to be only about 10-fold smaller. Thus it looks quite possible that the largest part of the polypeptide chain might well not be necessary for catalytic purposes but might be involved in structural interactions with synaptic elements, including for instance the tail of the electric organ enzymes.

# D. CATALYSIS

## 1. Catalytic mechanism

Acetylcholinesterase is a serine hydrolase along with other esterases and peptidases and shows irreversible active site phosphorylation (Cohen and Oosterbaan, 1963; Rosenberry, 1975a). The reaction takes place according to the equation:



The peptide chain in the region of that serine residue has been shown to be very similar to the active site of other enzymes within this classification such as chymotrypsin and elastase (Schaffer, Michel and Bridges, 1973). The structure of the active centre of AChE is very complementary to acetylcholine. The substrate has a quaternary nitrogen which is consequently positively charged and also an ester bond. The enzyme active centre correspondingly possesses a negatively charged "anionic site" which attracts the quaternary nitrogen and orientates the ester bond over an "esteratic site" which is responsible for the hydrolytic process (Wilson and Bergmann, 1950a; Krupka, 1964).

The hydrolytic process takes place in two stages. The enzyme combines with the substrate to form the enzyme-substrate (Michaelis-Menten) complex which is then hydrolysed to the final products. The catalytic mechanism of chymotrypsin has been shown to have a characteristic "charge-relay" system of hydrogen bonds comprising

the active site serine hydroxyl, the imidazole side chain of histidine and the carboxyl group of aspartate (Blow, Birktoft and Hartley, 1969). The acylation or deacylation by substrate of the enzyme shows general base catalysis by the imidazole (Bender, Clement, Kezdy, D'A Heck, 1964) and it is suggested that in the charge relay system the imidazole withdraws protons from the serine hydroxyl as the substrate acylates the enzyme. The analogy is drawn with AChE because it also shows an apparent $pK_A$ of between 6 - 7 which indicates general acid-base catalysis involving an imidazole group. Experiments on pH dependence of hydrolysis by AChE have shown that the enzyme relies on groups which ionize at pH 6.5 and 9.4 (Krupka and Laidler, 1960). These values are similar to those (pH 6.2 and 10.1) which are found during the reaction with saturating acetylcholine. The characteristic bell shaped curve of activity versus pH showing maximal activity between pH 8-9 indicates the dissociation of acidic and basic groups. Krupka (1967) has shown that the activity of AChE also depends on a second imidazole, the basic group of which has a $pK_A$ 5.6. He suggests that this imidazole ($pK_A$ 6.5) has the role of a general base in deacetylation and also stops cation binding at the active site when protonated. This mechanism is not generally favoured (Rosenberry, 1975b). Also, the view that the basic group with a $pK_A$ of 6.5 is the enzyme nucleophile has been discarded in favour of the serine hydroxyl (Froede and Wilson, 1970). A more likely mechanism is Brestkin and Rozengart's (1965) explanation of the model of Wilson, Bergmann and Nachmansohn (1950) (see Fig. I-6). The single imidazole group in the active site increases the nucleophilicity of the serine oxygen. A covalent bond is then formed between the oxygen and the carboxyl group in the acetylcholine and the choline is then

released. Deacetylation follows the reverse process as the acylated enzyme is hydrolysed.

Froede and Wilson (1970) explain the mechanism as follows:

$$E + S \rightleftharpoons E \,.\, S \rightleftharpoons E'P_1 \rightleftharpoons E' + P_1 \xrightarrow{H_2O} E'P_2 \rightleftharpoons E + P_2$$

where S = substrate

E.S = Michaelis complex

E'.$P_1$ = Michaelis complex. Acetyl-enzyme. choline

$P_1$ = choline

E' = Acetyl-enzyme

E'$P_2$ = Michaelis complex. Acetic + enzyme

$P_2$ = Acetic acid

when $P_1$ and $P_2$ are in concentrations low enough not to affect the kinetics, the dissociation of complexes is rapid.

$$E + S \underset{k_{-1}}{\overset{k+1}{\rightleftharpoons}} E.S \xrightarrow{K_2} E' + P_1 \xrightarrow[K_3]{H_2O} E + P_2$$

The Michaelis Menten steady state equation thus give the reaction velocity:

$$v = \frac{V\,max}{\frac{Km}{S} + 1}$$

## 2. Inhibition

Much information has been gained about the active site structure of AChE with the use of active site inhibitors (both anionic and esteratic site inhibitors).

### (a) Anionic site inhibitors

Wilson and Bergmann (1950a) first demonstrated the presence of a negative centre in AChE with the aid of competitive inhibitors

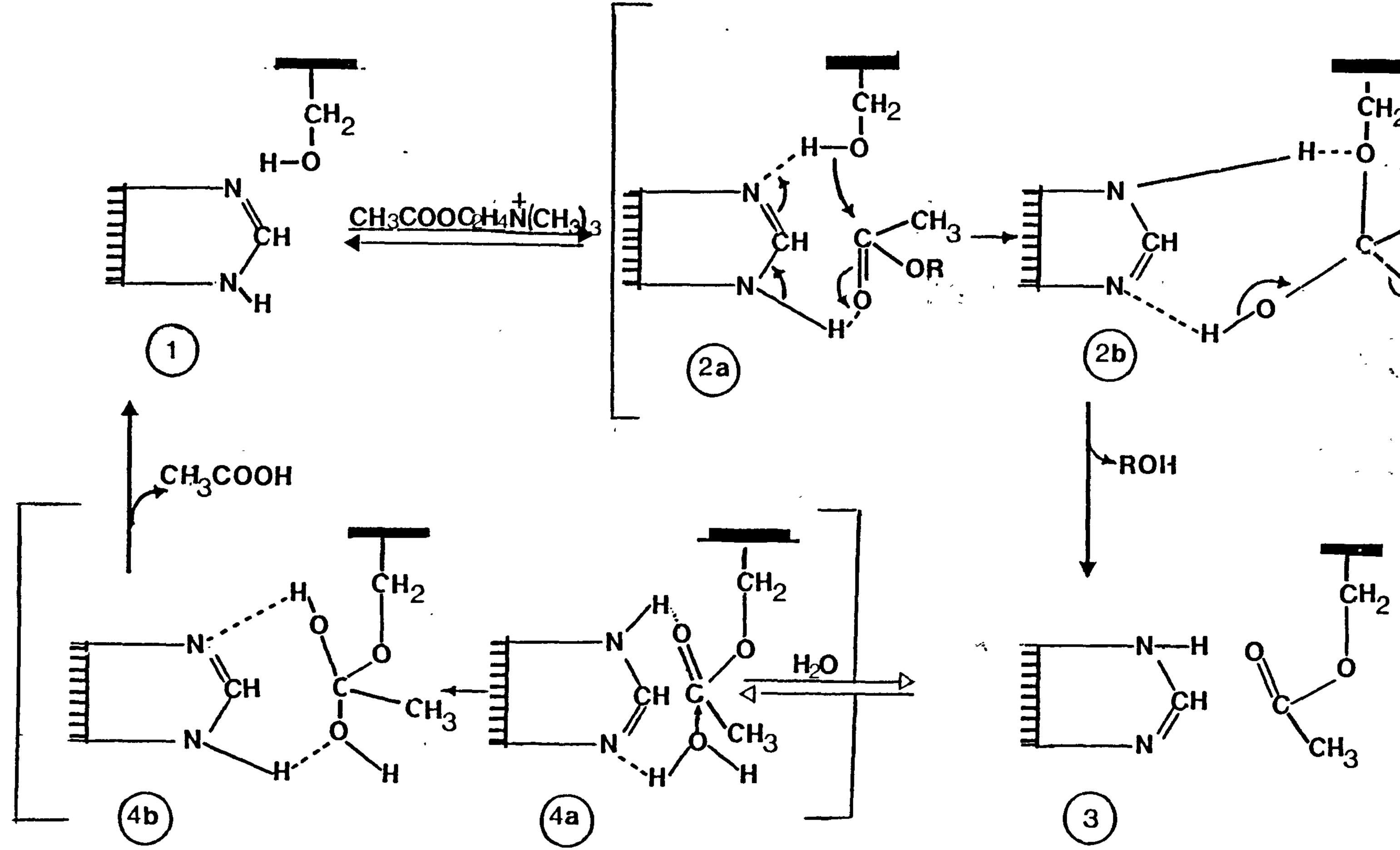
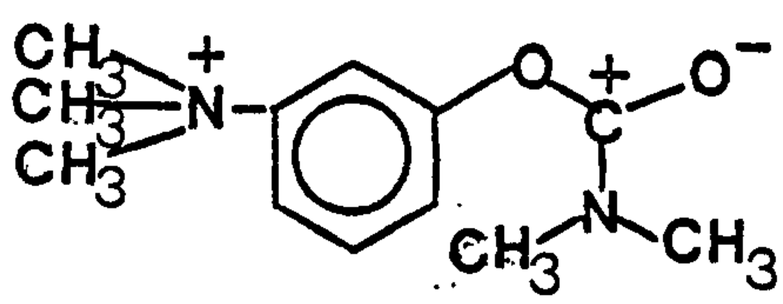
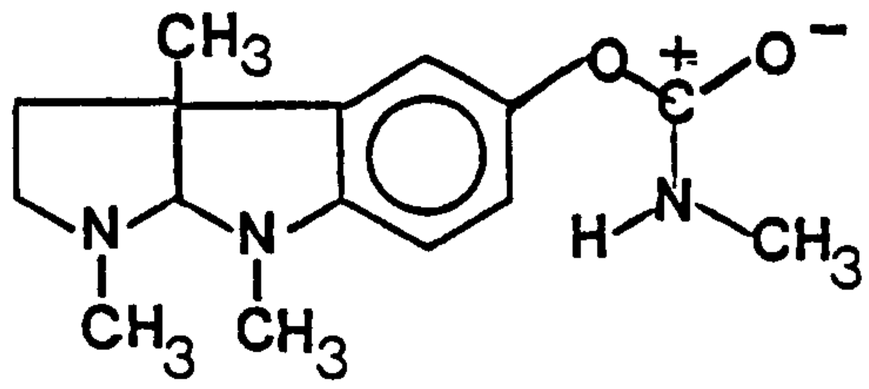



FIG. I-6. Acetylcholinesterase catalysis (Brestkin and Rozengart 1965)

Two competitive inhibitors of similar structure which they used were physostigmine and prostigmine. However, these two compounds differ in that prostigmine has a quaternary ammonium ion and consequently is positively charged at any pH; physostigmine is a tertiary amine and changes from conjugated acid at pH 6 to a neutral molecule at pH 10. Therefore physostigmine begins to lose its effectiveness as a ligand for the anionic site above pH 6 whereas prostigmine does not.

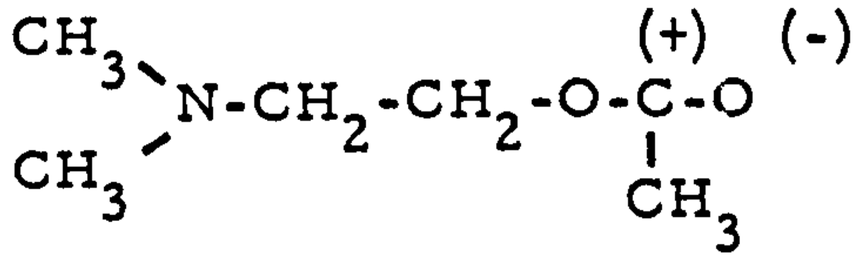


Prostigmine



Physostigmine

Hestrin (1949) and Wilson and Bergmann (1950b) showed a similar effect with substrates. Dimethylaminoethyl acetate is a conjugated acid below pH 8.3 and is consequently cationic whereas at higher pH values it is unchanged. Likewise it is hydrolysed rapidly by AChE at pH values between 8 - 9, but above this range it loses its effectiveness as a substrate. On this basis of the electric charge upon substrates and its effect on enzyme binding, the mode of binding acetylcholine to AChE has been accepted as ionic bonding to an anionic site.

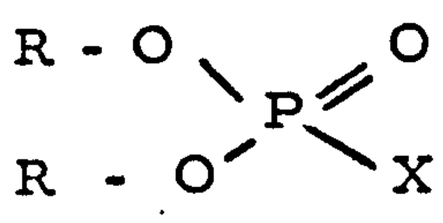
Dimethylaminoethyl acetate 

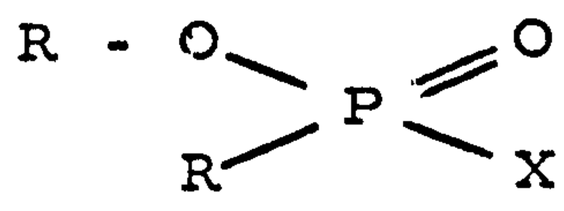
However Wilson (1952) showed that in addition to the above mentioned coulombic forces, unspecific Van der Waal's forces contributed to

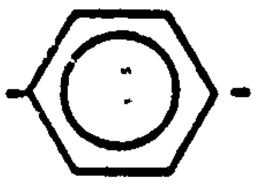
the binding of molecules to the anionic site. Using the competitive inhibitor hydroxyethyl-ammonium at pH 7 (cationic) he observed the effect of binding to the anionic site of sequentially methylating the nitrogen. He found that each alkyl group increased binding seven fold except for the fourth methylation. He reasonably assumed that this enhancement was due to Van der Waal's attraction by the methyl group to hydrocarbon moieties in enzyme. The fourth methyl group would have little effect due to the tetrahedral nature of the molecule stopping the group from being close to the enzyme.

b. Esteratic site inhibitors

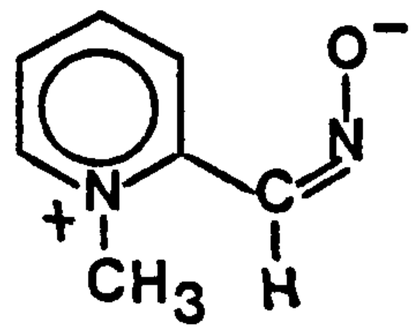
The process of catalysis at the esteratic site has already been discussed under the heading of 'Catalysis', and the point been made that the carbonyl group of acetylcholine binds covalently to the serine oxygen. Much understanding of the esteratic site has been gained by the use of organophosphate inhibitors, and has led eventually to the development of nerve gases, insecticides and consequently to antidotes. The mechanism of organophosphate inhibition is that the electrophilic phosphorus atom forms a covalent bond with a nucleophilic group in the esteratic site with the elimination of an acidic group such as $F^-$ (Wilson and Bergmann, 1950a). However, unlike the acetylated enzyme, the phosphorylated AChE is hydrolysed extremely slowly or not at all by water. Organophosphates have the general formula



where X = $F^-$, $Cl^-$, $CN^-$, $O_2N$-  -$O^{(-)}$ i.e. X = an acidic group.

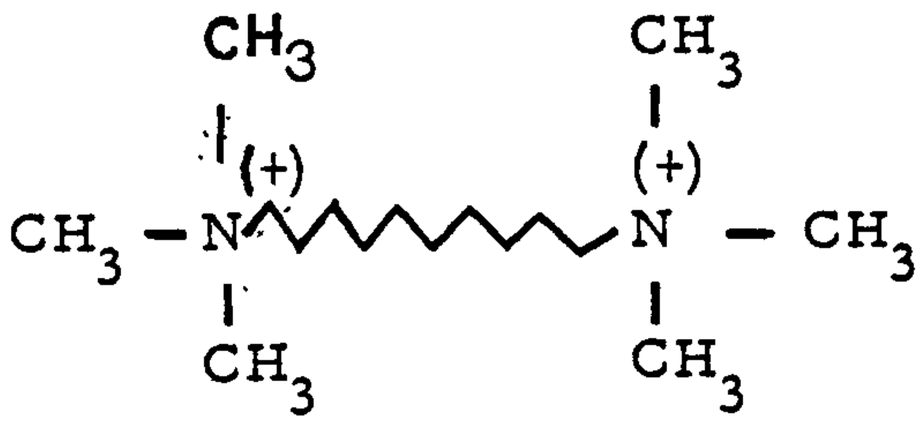
Organophosphates could be regarded as substrates as they bind to the esteratic site optimally at pH 8 which is the same as substrates (Wilson and Bergmann, 1950a). Wilson (1951) showed that hydroxylamine (a nucleophilic reagent) reactivated organophosphate inhibited AChE by 50% within 5 hours by competing for the electrophilic phosphorus atom in the esteratic site. He then suggested that if such a nucleophilic reagent could be linked to a cationic nitrogen which would bind to the anionic site of AChE, the reactivating power would be enormous. This led to the development of 2-pyridine aldoxime methiodide (2-PAM) which has found to reactivate AChE which had already been inhibited by DFP (Wilson and Ginsburg, 1955).



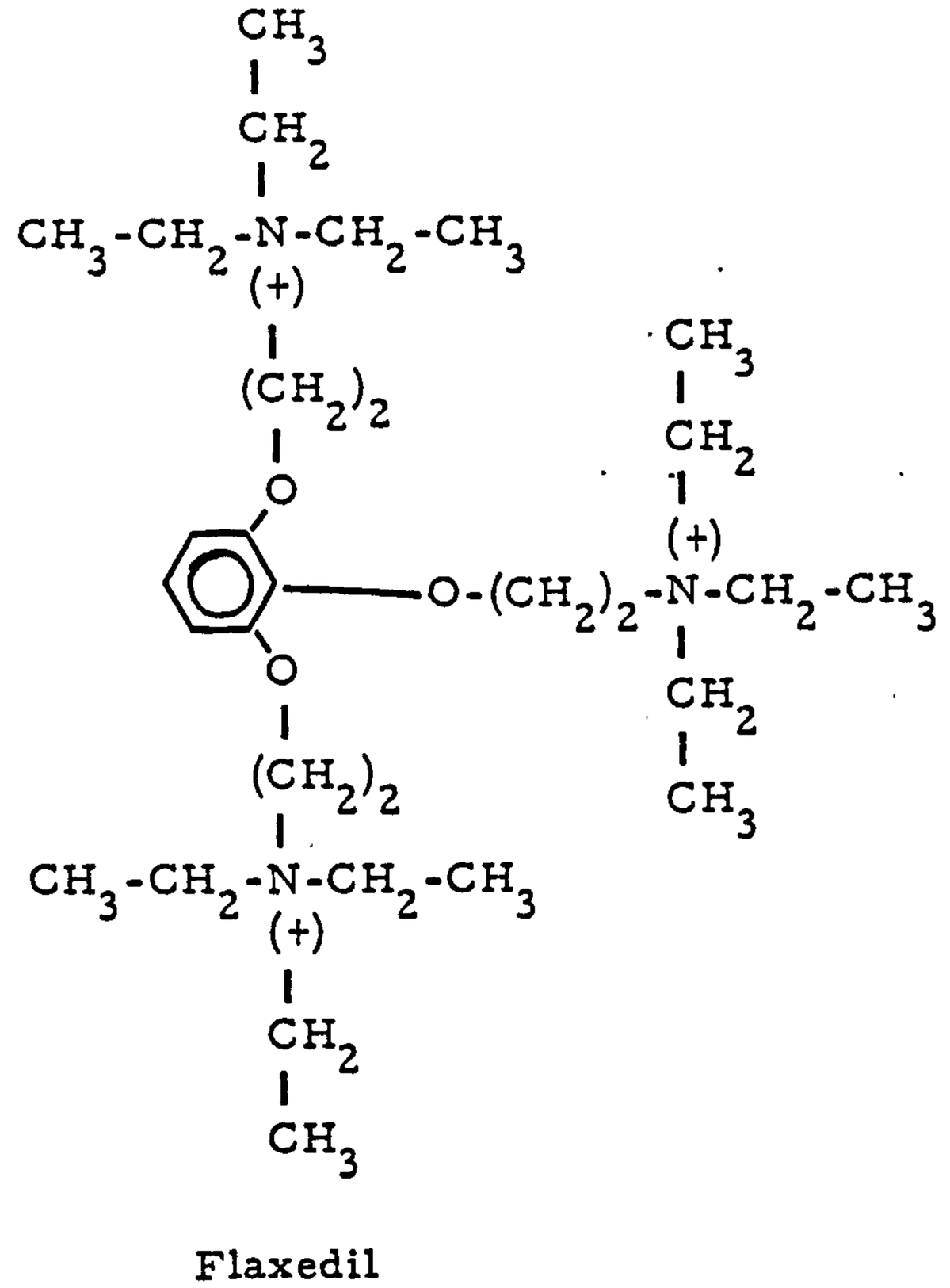
3. Peripheral Anionic Sites and Induced Fit

Recently evidence has accumulated indicating that acetylcholinesterase possesses, in addition to the anionic site in the catalytic centre, a peripheral anionic site where ligands bind and exert a regulatory role on the enzyme activity. The first evidence for such allosteric sites was presented by Changeux (1966) from experiments with neuromuscular blocking agents of the depolarizing and nondepolarizing types. Decamethonium, a depolarizing blocking agent with two cationic groups, has a high affinity for AChE at low ionic strength. The inhibition follows Michaelis-Menten kinetics and it is considered that one of the trimethylammonium groups binds to the catalytic site and the other to a peripheral anionic site,

(Changeux, 1966; Kitz et al, 1970). On the other hand, inhibition of the enzyme by the nondepolarizing blockers gallamine and d-tubocurarine does not follow simple competitive kinetics. This suggests that the substrate and inhibitor are bound simultaneously by the enzyme and implies the existence of a peripheral site (Changeux, 1966). In addition, it has been demonstrated that flaxedil (which has three quaternary nitrogens) binds tightly to AChE but binding is not antagonized by active site inhibitors (Changeux, 1966) or by monoquaternary ligands which bind to this peripheral site (Roufogalis and Quist, 1972).



Decamethonium



Flaxedil

The effects of small quaternary ions (Roufogalis and Thomas, 1970) and inorganic ions on acetylcholinesterase activity have been investigated and from these results Wilson (1970) has discussed these interactions as possibly being due to conformational changes in the protein molecule. In a study of the effects of $Ca^{+2}$, tetraethyl-

ammonium ion (TEA), tetramethylammonium ion (TMA), Flaxedil and decamethonium on substrate hydrolysis Roufogalis and Quist (1972) have built up a model with three anionic sites to explain their results. They consider that $Ca^{+2}$ must bind solely at a $\beta$-anionic site (allosteric site) which acts as an accelerator site. TEA binds at the $\beta$-anionic site but also interacts with the $\alpha$-site (the main anionic site) and decamethonium binds across the $\alpha$ and $\beta$-anionic sites. Flaxedil is postulated to bind at a third anionic site ($\Upsilon$) which can regulate the activity of the $\beta$ and catalytic sites.

Further evidence for an allosteric site has come from studies on displacement of the fluorescent inhibitor N-methyl acridinium from the active site by decamethonium and d-tubocurarine(Mooser and Sigman, 1972) and the existence of a site which binds indophenyl acetate and is topographically distinct from other binding sites (Chiu and O'Brien, 1972).

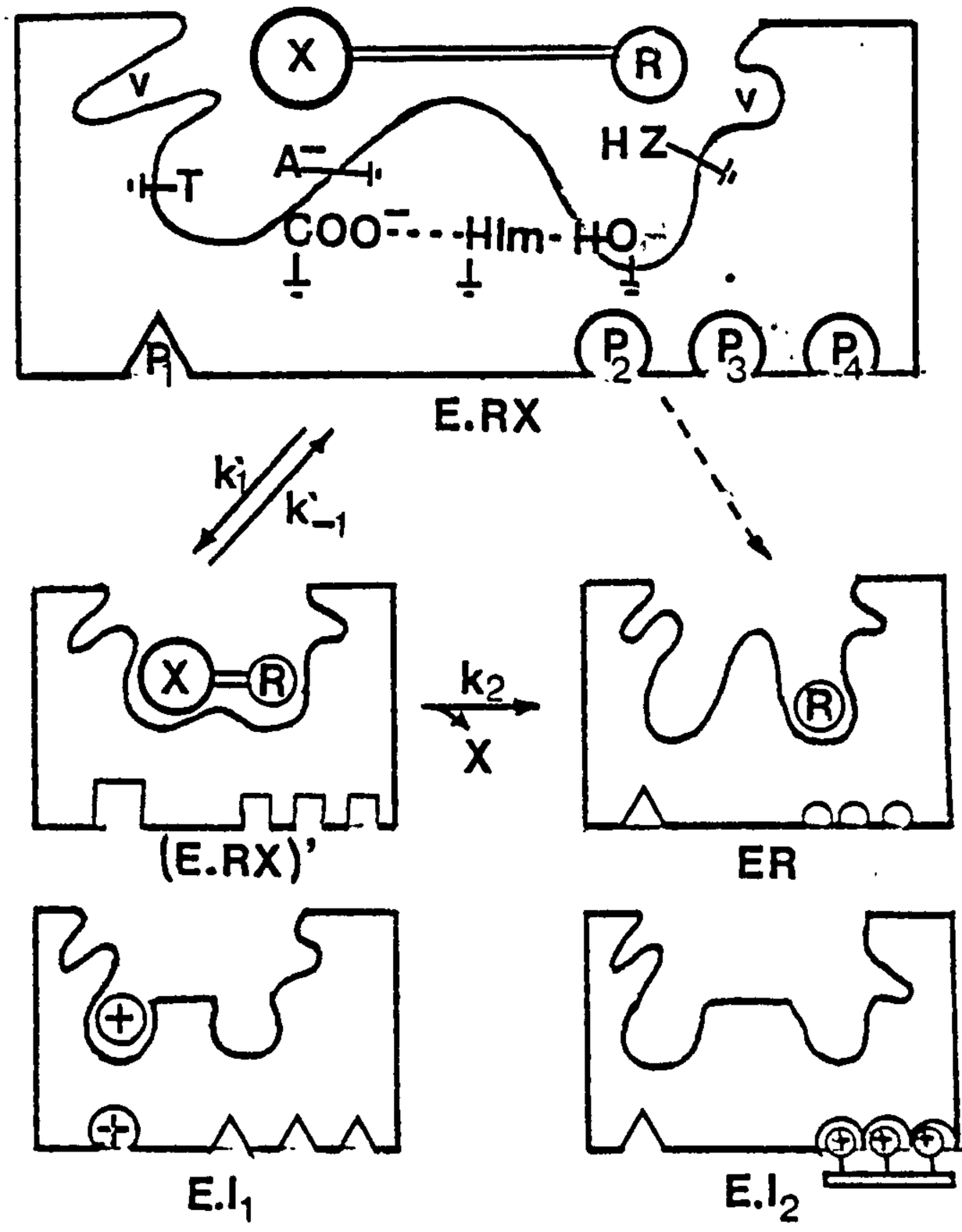
An interesting theory, to account for substrate inhibition has been recently proposed by Kato et al (1972). These workers showed that atropine and l-hyoscyamine interact at a region distinct from the catalytic site (Kato et al, 1970; Kato and Yung, 1971). Velocity versus substrate concentration curves with acetylthiocholine, in the presence of l-hyoscyamine, atropine or gallamine become sigmoidal at normal substrate saturated conditions. Kato et al (1972) interpret their results by assuming Monod's model and postulating that with high substrate concentrations conversion from R to T occurs and this has reduced catalytic activity and binds atropine. Atropine competes with substrate at the allosteric site and therefore relieves inhibition.

A few workers have studied allosteric properties of the membrane bound enzyme. Wins et al (1970) concluded, from the effects of divalent cations on d-tubocurarine inhibition of membrane bound torpedo AChE, that a non catalytic site acts in the membrane bound enzyme.

On the basis of these observations, Rosenberry (1975b) has formulated an "induced fit model" for AChE (Fig. I-7) based on the induced fit model of Koshland (1958). The enzyme substrate complex E-RX is assumed to be in equilibrium with free E and RX and conformations of the catalytic site are similar for the free enzyme and in E-RX. The E-RX complex reaches for the induced fit complex $(E\text{-}RX)^{'}$ conformation which increases acylation by lowering the transition state for the covalent bond rearrangement in $K_3$. He suggests that reversible ligands which bind to peripheral sites modulate activity by reducing conformational flexibility and stabilizing catalytic site conformation; this reduces the transition state for acylation by poor substrates but varies it for good substrates. It is too early to judge the general opinion of this model, but it does serve to explain the functions of the anionic sites.

FIG. I-7

Induced Fit Mechanism for AChE (Rosenberry, 1975b)




The initial enzyme substrate is complex E.RX; the induced-fit complex, $(E.RX)^{1}$; and the acyl enzyme, E.R. The enzyme ligand complex with bisquaternary ligands like decamethonium ($E.I_1$) involves the anionic site and peripheral site Pi; the complexes formed by other multiquaternary ligands like flaxedil ($E.I_2$) involve ligand binding at other peripheral sites ($P_2, P_3, P_4$). Identified residues at or near the catalytic site include the charge-relay complex ($COO^-$ --- HIm ... HO); an acidic group HZ; the anionic group $A^-$ which defines the anionic site; a tryptophan residue T near the anionic site; and adjacent hydrophobic areas V.

# E. THE PHYSIOLOGICAL SIGNIFICANCE OF ACETYLCHOLINESTERASE

## 1. Conduction of the Nervous Impulse

### a. Hodgkin-Huxley Theory

The first description of nerve excitation in terms of a sequence of time and voltage-dependent ionic conductance changes was given by Hodgkin and Huxley (1952). The original hypothesis is still valid today and most of the more recent work has endorsed the physical events which were proposed to account for the conduction process.

Both nerve and muscle cells maintain a potential difference across their surface membrane which is caused by the unequal distribution of ions. The cell interior is rich in $K^+$ ions and the extracellular fluid is rich in $Na^+$ and $Cl^-$ ions. As a consequence the interior of the cell is maintained at a negative potential of -50 to -100 mv with respect to the exterior. The steady state of the excitable cell is maintained through the utilization of metabolic energy in driving a $Na^+.K^+$-ATPase that serves to expel accumulated $Na^+$ from the interior and accumulate $K^+$ from the exterior (Katz, 1966). Since the $K^+$ (and $Cl^-$) conductances are greater than the $Na^+$ conductance, a potential difference is built up and maintained across the cell membrane. In electrical terms the membrane acts as a leaky resistance to the three ions and each is subject to independent control. Each pathway is defined by a characteristic equilibrium potential that represents the potential difference necessary to balance the tendency of ions to diffuse down its activity gradient.

The observed membrane potential lies between the values for $Na^+$ and $K^+$ and because of the greater $K^+$ conductance, is closer to the equilibrium potential for $K^+$.

A nervous impulse may be initiated by transient depolarization of the membrane and a lowering of the membrane potential beyond a certain unstable point (the threshold) leads to an automatic transient reinforcement of the initial displacement (the spike). Ionic contributions to the spike potential involve an initial rapid increase in the $Na^+$ conductance that is converted within m. sec. into an increased and opposing $K^+$ conductance.

Despite the success of this theory, it does not offer any physicochemical description of the molecular basis for the ionic events, although available data suggests that $Na^+$ and $K^+$ channels or pores exist and that these are separate entities. Among the evidence is the selective blocking of $Na^+$ conductance by tetrodotoxine and of $K^+$ chanels by quaternary ammonium ions. There is also evidence to suggest that membrane proteins are involved in the selective transport of $Na^+$ and $K^+$ ions through the channels and that these have a dumb-bell shape, with two hydrophilic ends linked by a central section possessing lipophilic side chains, but with a hydrophilic core (Keines, 1972).

Implicit in the Hodgkin-Huxley theory is the concept of a conformational change in the membrane. Optical techniques have been applied to measure membrane changes in birefringence, light scattering, and fluorescence emission of the membrane after staining with fluorescent dyes. However these have not been notably successful (Keines, 1972). Evidence for a conformational change

has been claimed by Clark and Strickholm (1971) from measurements of the pH dependency of crayfish axon cable impedence. They interpret their results as showing co-operative transition in the surface membrane proteins.

Other questions, on the 'gating mechanism' and the involvement of $Ca^{++}$ still await answers but it is likely that phospholipid molecules are also involved. The flexible polar groups may alter their position thus opening or closing 'gates' and Armstrong and Bazanilla (1973) have provided some evidence for 'gating currents' produced by this movement.

An alternative model to explain the generation of the action potential has been developed by Tasaky, (1968). In this scheme, the membrane is visualized as acting as a cation exchanger and changes in the cation concentration result in its conversion from a relatively impermeable state to a highly permeable state. The driving force for the conversion is the displacement of externally bound calcium by monovalent cations.

b. Nachmansohn's Theory

In a series of articles, Nachmansohn (1953-1971) has formulated a hypothesis which, although controversial, attempts to explain a number of facts which are not considered in the Hodgkin-Huxley theory.

These unexplained observations may be outlined as follows:

1. High concentrations of AChE are present in axon fibres as well as at synaptic regions.

2. The turnover time of acetylcholine (30-40 μsec.) is sufficient

to account for the rapid changes involved in conduction.

3. There is a coincidence between the time when nerve cells begin to function, during growth, and the time when high concentrations of AChE appear.

4. In the electroplax of electric eel there is a direct correlation between the concentration of AChE and the voltage produced.

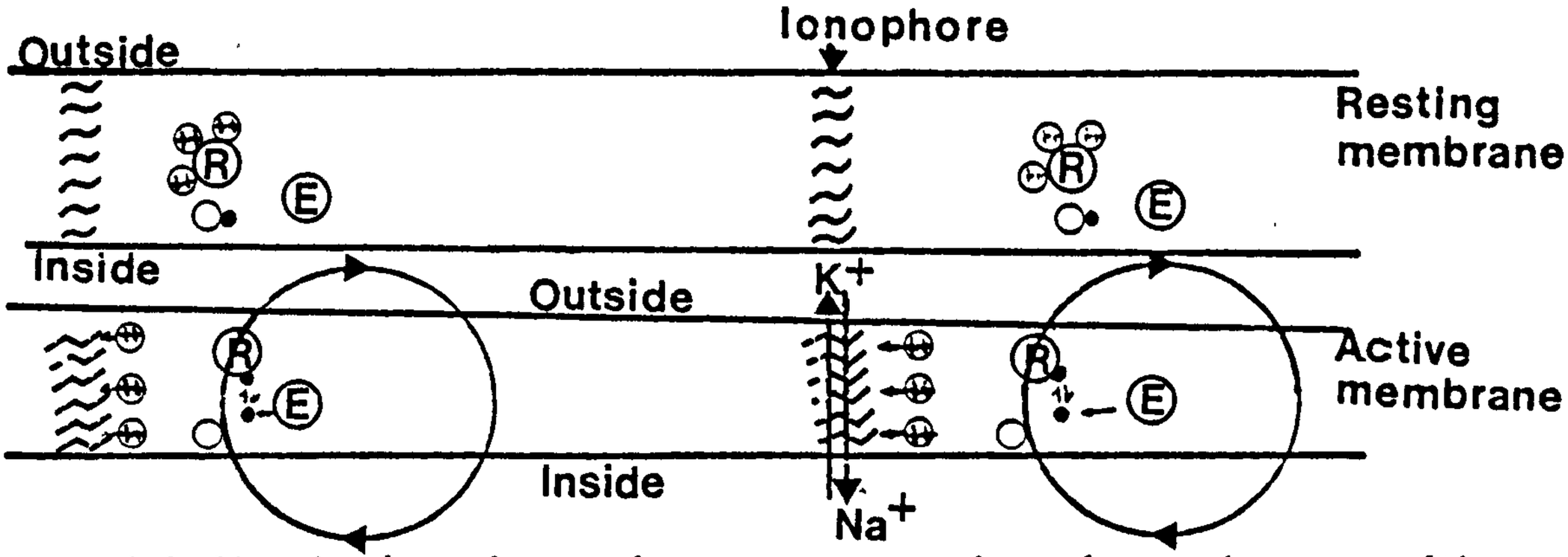
5. DFP blocks axonic conduction and concurrently inhibits AChE. With sensitive techniques it may be demonstrated that as long as conduction is maintained AChE is present.

6. Axons exposed to organophosphates lead to irreversible block of electrical activity and 2-PAM may restore the activity.

7. The presence of an acetylcholine-receptor (see later) has been demonstrated in many axonal membranes. Provided structural barriers to lipid insoluble quaternary nitrogen derivatives are either not present or are removed (e.g. by lysolecithin), acetylcholine or d-tubocurarine interact with the receptor.

8. Local anaesthetics (lipid soluble acetylcholine-receptor inhibitors) block electrical activity.

Although the methodology in some of the experiments from Nachmansohn's laboratory have been questioned (O'Brien, 1960; Koelle, 1963; Karczmar, 1967) the key rôle postulated for the receptor and AChE is of considerable interest, and an outline of the model is given below.




Acetylcholine is the trigger that starts a series of reactions resulting in a permeability change of the excitable membrane. Release and the action of ACh are taking place <u>within</u> the membrane. In the resting condition ACh (•) is bound in a storage form. On excitation ACh is released and acts on the receptor (R) inducing a conformational change which may be allosteric and causes the release of calcium ions (++) bound to protein. These ions induce a conformational change in phospholipids and other polyelectrolytes thus permitting accelerated ion movements. Amplification of the signal initiates a new electrical circuit that stimulates adjacent points and the process is repeated. ACh is then rapidly hydrolysed by AChE(E), the receptor returns to its resting condition and the barrier for ions is re-established.

## 2. Synaptic Transmission

Transmission of the nervous impulse across a synapse may be controlled by either electrical or chemical processes. The former has so far only been detected in invertebrates and some lower vertebrates but may be shown to have more general significance in the future. At most junctions chemical transmission takes place via the release of a transmitter from the presynaptic terminal

and an induced permeability change in the postsynaptic membrane caused by a specific transmitter-receptor interaction. At some synapses the effect of the transmitter is to cause an activation of a metabolic system in the postsynaptic element without increasing the ion permeability of the postsynaptic membrane (Weight, 1971).

In the generally accepted theory of chemical transmission, the sequence of events may be broken down into a number of stages (Fig. I-8).

a. Neuronal uptake of precursors of the transmitter or the transmitter itself.

b. Synthesis and storage of transmitter in the nerve endings.

c. Liberation of the transmitter (excitatory or inhibitory) on the arrival of the nerve action potential (NAP).

d. Interaction of the excitatory transmitter with postsynaptic receptors to produce a localized depolarization, the excitatory postsynaptic potential (EPSP), through an increase in permeability to ions. The inhibitory transmitter causes a selective increase in permeability to the smaller ions ($K^+$ and $Cl^-$ chiefly) resulting in a localized hyperpolarization, the inhibitory postsynaptic potential (IPSP). The EPSP initiates a conducted NAP, in the postsynaptic membrane; this can however be prevented by the hyperpolarization induced by a concurrent IPSP. In some synapses intracellular effectors may be increased (e.g. noradrenaline, serotonine, and histamine can increase cyclic AMP levels).

e. Dissipation of the transmitter by enzymatic action, by diffusion, or by reuptake into the axonal terminal.

Apart from acetylcholine, a number of biogenic amines

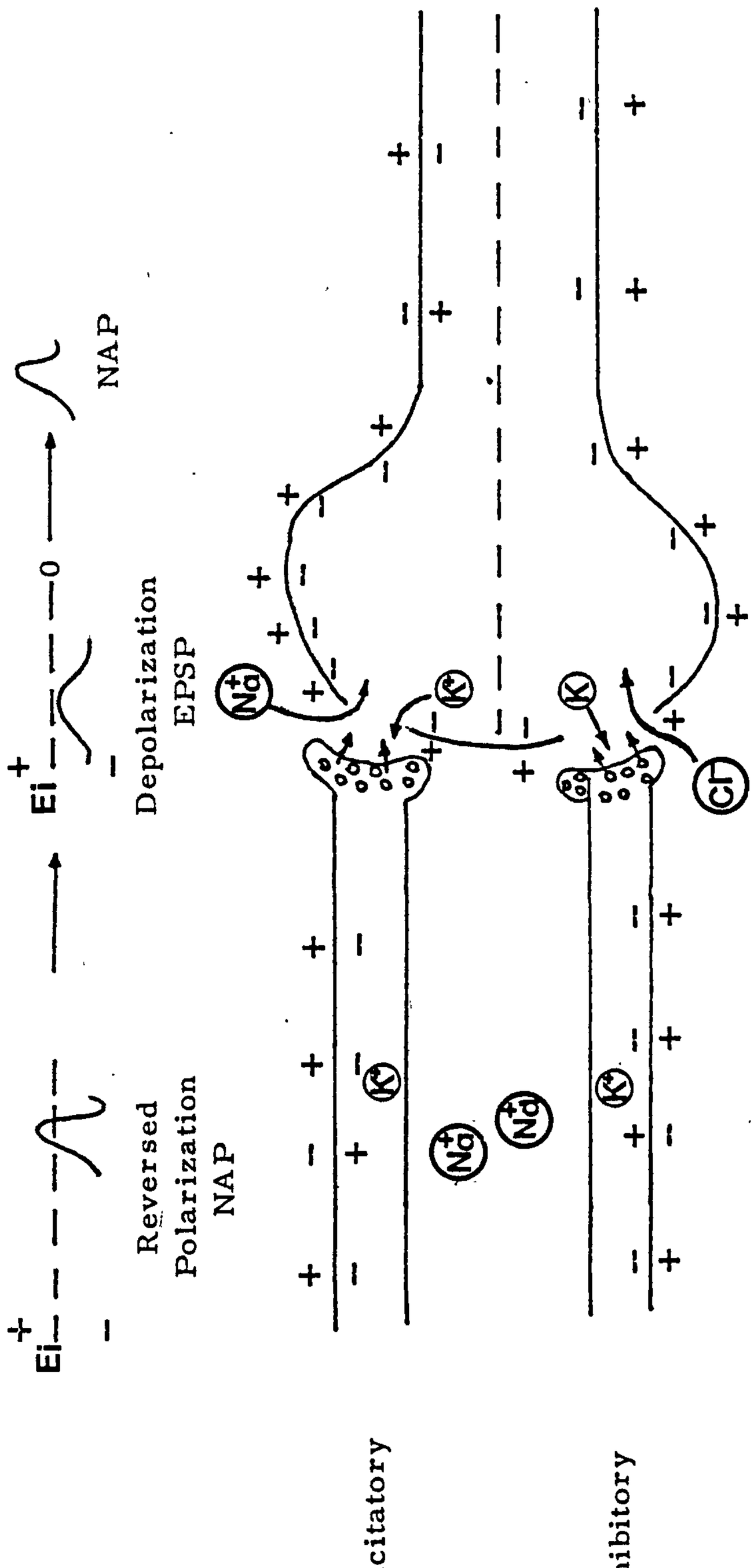



Fig. I.8
Steps involved in Excitatory and Inhibitory Synaptic Transmission (from Koelle, 1971)

(noradrenaline, dopamine, 5-hydroxytryptamine) and amino acids (glycine, glutamic acid, γ-aminobutyric acid, taurine) have been put forward as putative neurotransmitters but the case for ergothioneine seems tenuous (Briggs 1972). Only the postsynaptic events will be considered in certain depth although a brief outline of the synthesis and release of acetylcholine is necessary to make this meaningful.

a. Synthesis and release of acetylcholine

Acetylcholine is synthesised in the neuron by the enzyme cholineacetyltransferase (acetyl CoA - choline O-acetyltransferase from the precursors acetyl~CoA and choline.

Neuronal uptake of choline is affected by a saturable carrier-mediated transport system which is $Na^+$-dependent and inhibited by hemicholinium-3 (HC-3) (Schubert et al, 1966; Potter, 1968; Marchbanks, 1968). The uptake system is able to keep up with acetylcholine release (Potter, 1970) and appears to be accelerated by electrical stimulation. It seems unlikely that acetylcholine uptake occurs to any appreciable extent under physiological conditions (Katz et al, 1973).

The origin of acetyl-CoA is uncertain. Most acetyl-CoA synthesis occurs intramitochondrially and since glucose and pyruvate stimulate ACh synthesis, it is probably derived from this source (Hebb, 1972). In any case, citrate and acetate may be converted to acetyl-CoA extramitochondrially by ATP citratelyase and acetyl-CoA synthetase respectively (review: Hebb, 1972).

With respect to the enzyme cholineacetyltransferase Fonnurn (Review 1973) has shown that it possesses a strong positive surface charge and also exists in different molecular forms. Under physiological conditions it is probable that the enzyme exists in soluble forms although localized binding to membranes cannot be ruled out.

In most present theories of transmission ACh is envisaged as being 'packaged' in synaptic vesicles in the nerve terminal (Hubbard, 1970, 1971; Israel et al, 1970) probably in a protein bound form, called by Whittaker synaptosomes (Whittaker, 1971, 1972) but with a nucleotide (possibly CMP) involved in the binding (Smythies et al, 1971).

Transmitter release occurs in the form of uniform packets (quanta) consisting of a relatively large number of molecules ($10^4$-$10^5$).

The release occurs spontaneously at a low rate and is enormously but transiently accelerated by a nerve impulse in a $Ca^{+2}$ dependent process (review: Katz, 1966). The release mechanism probably involves an exocytotic process in which vesicles discharge their contents into the synaptic cleft and are then refilled. Frozen preparations of Torpedo electroplax show vesicles attached to the membrane and open to the synaptic cleft (Nickel and Potter, 1971). Heuser and Reese (1973) have indeed shown micrographs of vesicles pinocytosing with the membrane. However, when AChE is injected into the axon which destroys the soluble ACh but not the occluded vesicular ACh transmission is abolished (Tauc et al, 1974). This evidence favours ACh release from the soluble fraction.

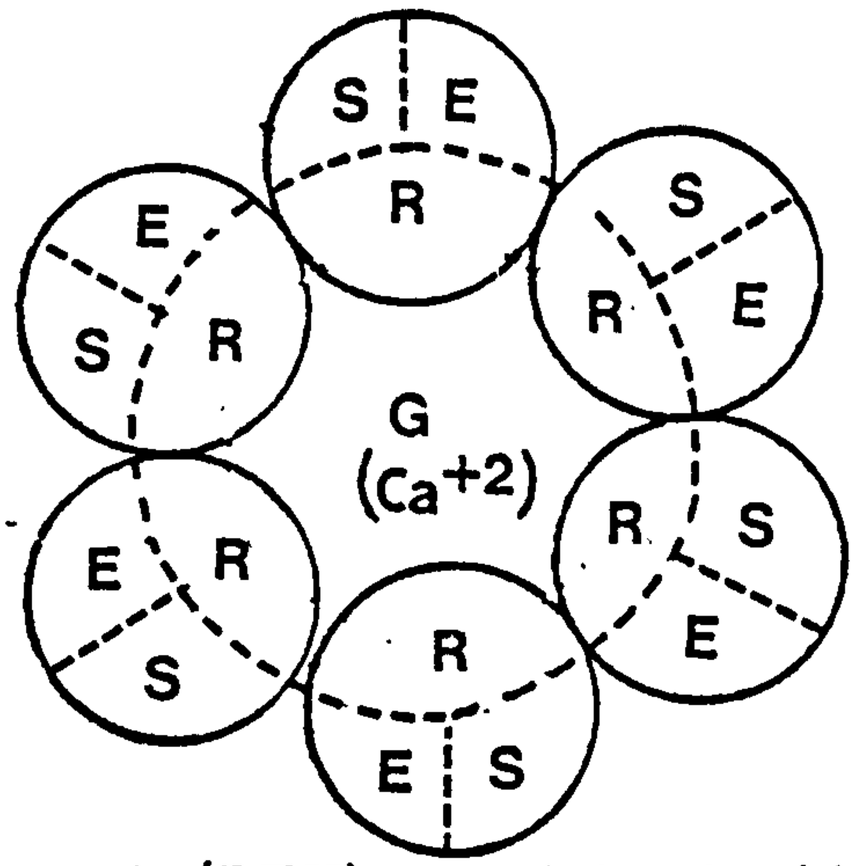
Further investigation is needed to elucidate the correct mechanism of ACh release. For a critical review of the origin of synaptic ACh, see Marchbanks (1974).

b. The postsynaptic response and termination of acetylcholine action

There are at least two basic units required to control the postsynaptic response to acetylcholine : the acetylcholine-receptor and AChE. The fact that both coexist in the excitable membrane indicate a very close relationship between the two glycoproteins and several authors still maintain that it has not been definitively disproved that they are one and the same molecule (Heilbronn, 1975). However, Simantov and Sachs (1973) have shown that there are large immunological differences between AChE and the receptor molecules which indicate they are in fact separate entities. The number of receptors and AChE sites have been shown to be equal in both membrane preparations (O'Brien et al, 1970) and solubilized fractions (Changeux et al, 1970). This stoichiometry is one of the reasons that led Neumann and Nachmansohn (1973) to formulate the "basic excitation unit" theory (BEU) (Fig. I-9). The various proteins of the acetylcholine cycle (Fig. I-10) have been rationalized into an "integral model" by Neuman et al (1973) in which the storage, receptor and enzyme proteins are combined into a basic excitation unit which surrounds an ion gateway. The fundamental novelty of this system is that acetylcholine is processed continually through the BEU's within the excitable membrane. This intramembrane concept contrasts with the widely held intracellular neurotransmitter

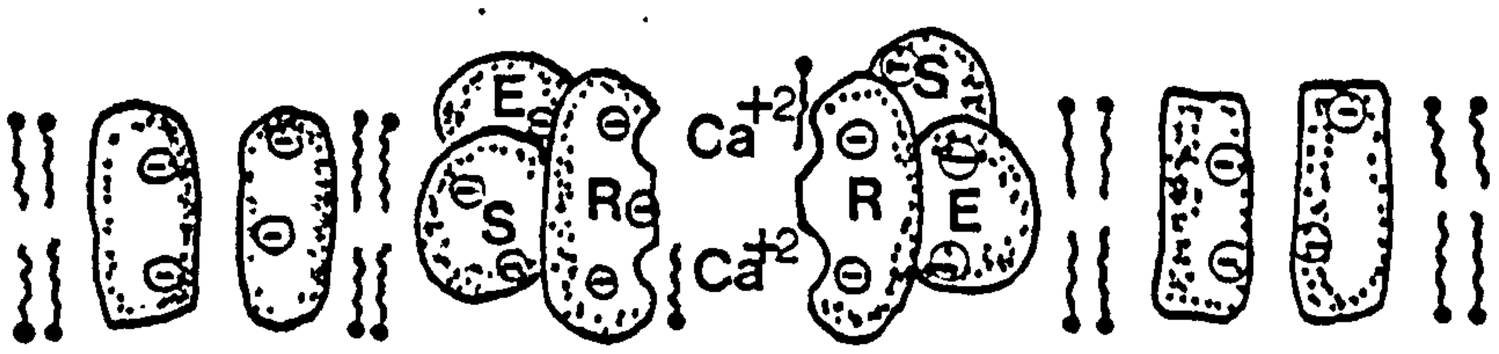
FIG. I-9

Schemes of AChE Controlled Gateway




Basic excitation unit (BEU) containing in this example 6 storage receptor/enzyme (SRE) assemblies viewed perpendicular to the membrane surface.

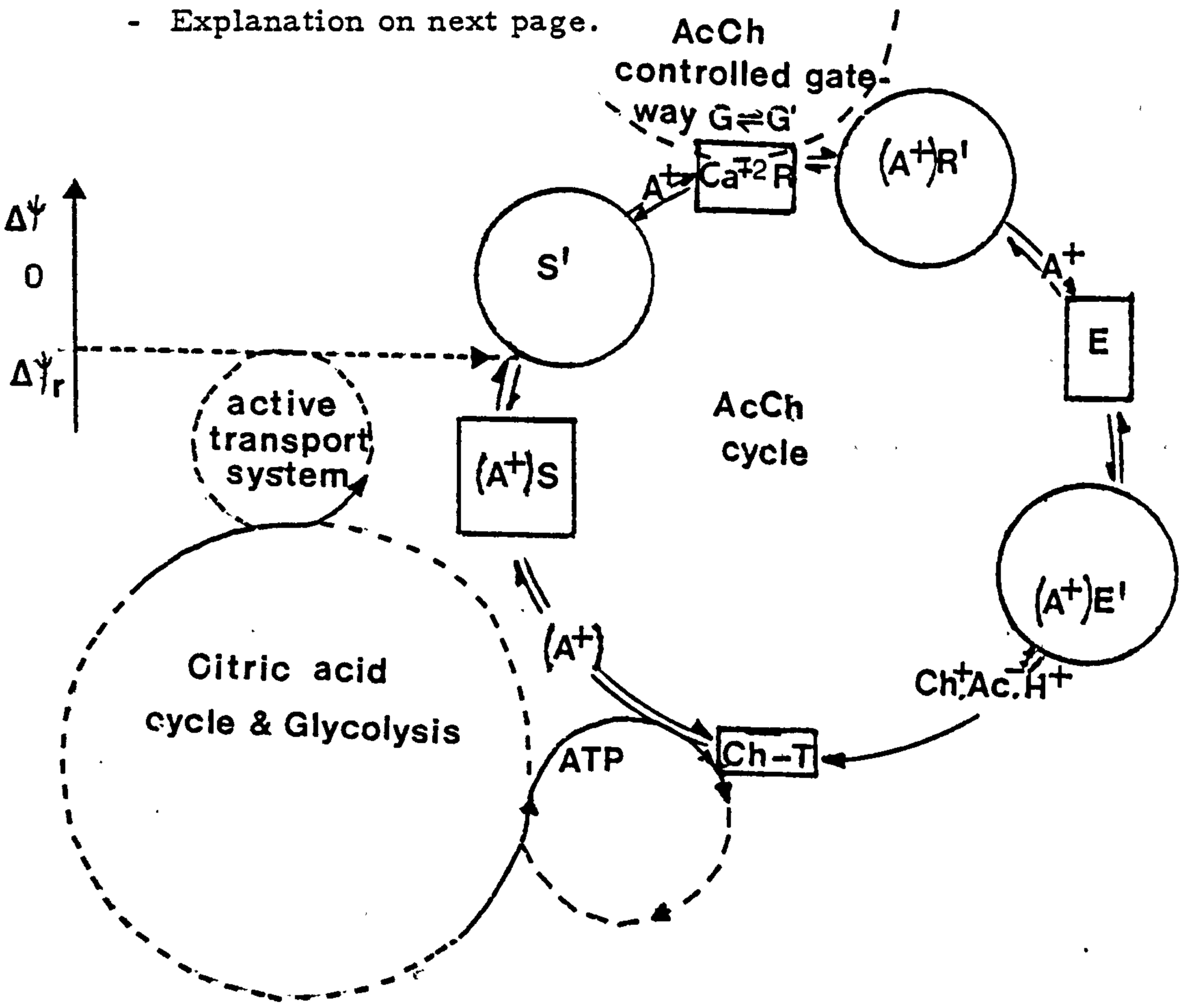
R = Receptor
S = Storage protein
E = Acetylcholinesterase
G = Gateway




Cross section through a BEU flanked by 2 units with model ion passages for $K^+$ ions in the resting stationary state. The minus signs (-) symbolize negatively charged groups of membrane components. (After Neumann. See Nachmansohn and Neumann, 1975).

Fig. 1.10 Acetylcholine Cycle (Nachmansohn and Neuman 1975)

- Explanation on next page.




$(A^+)$ = Acetylcholine; $(A^+)S$ = Acetylcholine-storage protein complex

$S'$ = empty storage protein. $(Ca^{+2})R$ = = Calcium dependent-receptor protein

$A^+R$ = Acetylcholine-receptor complex

E = Acetylcholinesterase

$(A^+)E$ = Enzyme-substrate complex.

G = Gateway closed; $G'$ = Gateway open

$\Delta\psi r$ = resting membrane potential $\Delta\psi$ = membrane potential

Explanation Fig. I-10

The binding capacity of the storage site for AcCh is assumed to be dependent on the membrane potential $\Delta\Psi$ and is thereby coupled to the active transport system and the citric acid cycle and glycolysis. The control cycle for the gateway G ($Ca^{+2}$ binding and closed) and the G' (open) comprises the SRE assemblies (see Fig. 1-9) and the choline O-acetyltransferase (Ch-T); Ch-T couples the ACh synthesis cycle to the translocation pathway of ACh through the SRE assemblies. The continuous subthreshold flux of ACh through such a subunit is maintained by the virtually irreversible hydrolysis of ACh to choline ($Ch^{+}$), acetate ($AC^{-}$) and protons ($H^{+}$) and by steady supply flux of ACh to the storage form by the synthesis cycle. In the resting stationary state, the membrane potential ($\Delta\Psi$ r) reflects dynamic balance between active transport (and ACh synthesis) and the flux of ACh (through the control cycles surrounding the gate-way) and of the various ions unsymmetrically distributed across the membrane. Fluctuations in membrane potential (and exchange currents) are presumably amplified by fluctuations in the local ACh concentrations maintained at a stationary level during the continuous translocation of ACh through the cycle.

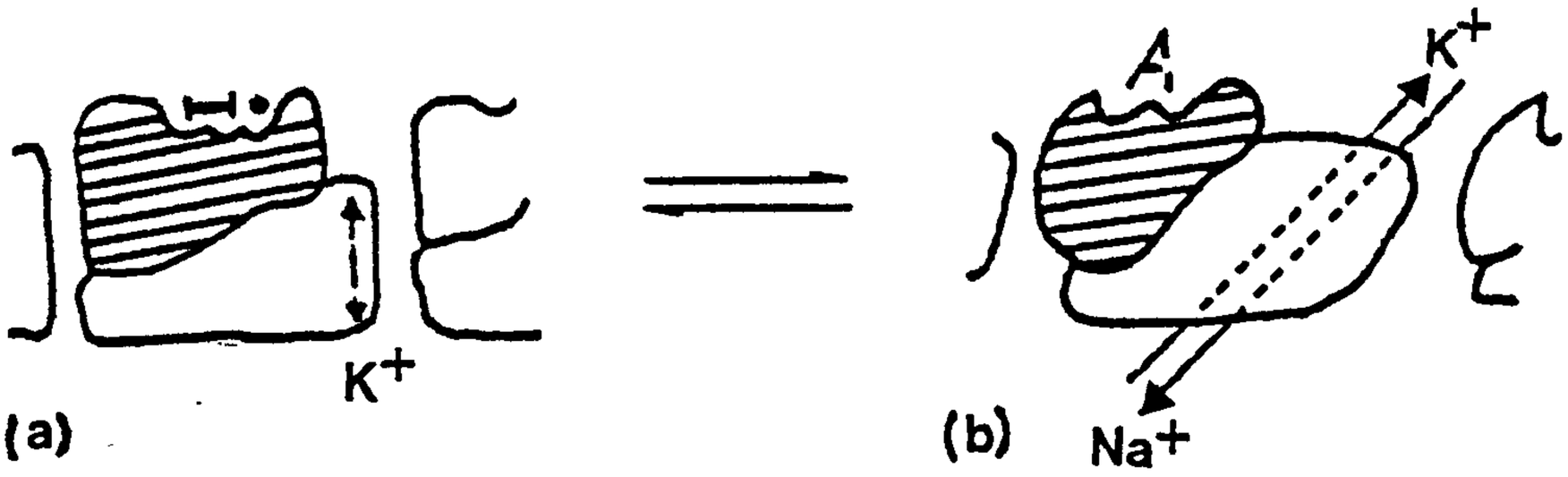
theory. In the integral model the generation of nerve impulse is brought about by a co-operative increase in the rate of ACh translocation through the BEU's. The model predicts that AChE is essential for the maintenance of the action potential in the membrane. The integral model brings together several facts and hypotheses in a controversial but attractive scheme which has already been questioned (Rosemberry, 1975). For a comprehensive review of the theory, see Nachmansohn and Neumann(1975).

(i) The acetylcholine receptor. In order to fulfil its function, the receptor macromolecule has to be intimately bound to, or built into the lipoprotein of the membrane. It must also have a high affinity for binding endogencus transmitter and exhibit a specific competition with drugs that act as agonists (activators that block electrical activity and simultaneously depolarize the membrane, e.g. acetylcholine, neostigmine, carbamylcholine, decamethonium) or antagonists (inhibitors that block electrical activity but without depolarization as d-tubocurarine and also local anaesthetics such as procaine and tetracaine).

To account for the translocation of ions through the membrane a reversible change in the macromolecule, in response to transmitter is proposed to occur (De Robertis, 1971). Podleski and Changeux (1970) postulate the existence of an acetylcholine protomer composed of an acetylcholine receptor (AChR) for acetylcholine recognition and an acetylcholine-ionophore (AChI) for ions translocation.

The measured response of monocellular electroplax preparations to receptor agonists and antagonists led to Changeux and

and Podleski (Podleski and Changeux, 1970) to develop a model of the protomer similar to the classical two states model for allosteric proteins (Monod et al, 1965)




(a) (b)

shaded region = ACh-receptor

Remainder of ACh-promoter = ACh-ionophore

(a) - resting state stabilized by inhibitors (I).

(b) - depolarized state stabilized by activators (A)

The protomer is postulated to exist in at least two conformations with R $\rightleftharpoons$ S in reversible equilibrium. R corresponds to the resting state of the membrane, largely permeable to $K^+$ which shows preferential affinity for and is stabilized by antagonists like -tubocurarine or p-(trimethylammonium) benzenediazonium. "S" corresponds to a depolarized state which is highly permeable to both sodium and potassium ions and shows preferential affinity for and is stabilized by agonist like ACh or decamethonium. Activation occurs through a reversible shift of the conformational equilibrium to the S state.

De Robertis' group (review: De Robertis, 1971) isolated proteolipids by chloroform/methanol extraction of synaptic junctions and electroplax membranes followed by Sephadex LH-20 chromatography. These proteolipids showed receptor characteristics and molecular weights of 40,000 and were distinct from AChE. Since then, the AChR protein has been extracted and purified in sizeable

amounts from the electric organ of a variety of fish: Electrophorus electricus (Changeux et al, 1976), Torpedo marmorata (Heilbronn and Mattson, 1974). T. californica (Claudio and Raftery, 1977) and others. Successful purification has also been achieved with other tissues: denervated adult skeletal muscle (Dolly and Barnard, 1975) and embrionic skeletal myotubes in culture (Merlie et al, 1978)

The "in vitro" characterization of the AChR protein received a considerable impetus from the introduction of two categories of ligands that make highly specific and stable complex with the AChR site and can be radiolabelled without losing their pharmacological activity: (a) the $\alpha$-toxins from the venom of a variety of snakes are compact polypeptides with 61-74 aminoacids and a flat shape revealed by X-ray crystallography (Low et al, 1976); they bind noncovalently to the AChR site with $K_D$ as low as $10^{-11}$ M and have a slow reversibility. (b) Affinity labeling reagents establish a covalent bond in or near the AChR-site (Karlin, 1969); a particularly efficient one is 4-(N-maleimido)-benzyltrimethylammonium iodide (MBTA) which reacts with a thiol group of the AChR site after exposure to the disulfide reducing agent dithiothreitol (Karlin, 1977).

SDS polyacrylamide gel electrophoresis of pure fractions of AChR-rich membranes gave distinct bands of apparent molecular as follows: 40,000, 43,000, 50,000 and 66,000 with T. marmorata and occasionally a band of 100,000-200,000 is observed in preparations of low specific activity (Sobel et al, 1977). Particularly pure preparation from T. marmorata showed only two major components of 40,000 and 43,000. The AChR is deeply integrated into

the membrane and detergents are needed to release it into solution. Non ionic detergents - Triton X-100, Tween 80 and Brij 35 - and negatively charged ones, such as deoxycholate or cholate, gave efficient solubilization without loss of α-toxin and cholinergic ligand bindings. Solubilization has also been achieved without detergents by extensive dialysis of E. electricus membrane fragments against a low ionic strength buffer followed by controlled tryptic digestion (Aharonov et al, 1975).

The most commonly used method for purification has been affinity chromatography with a cholinergic ligand coupled covalently to a solid matrix. The ligands selected were snake α-toxins and a variety of quaternary ligand derivatives.

The specific activity of the best preparations ranges between 8 and 12 μmole of α-toxin sites per g of protein (For Review see Heidmann and Changeux, 1978). The molecular weight per α-toxin site would be close to 100,000 but cannot be taken as the exact value of the protein mass per α-toxin site because of possible interference of detergent with protein determination.

Studies of the hydrodynamic properties of the detergent extracted protein are complicated by the presence of detergents and by the presence of different states of aggregation of the molecule. Sedimentation in sucrose density gradient of detergent crude extracts of purified AChR from Torpedo reveals two major components: a light form (L) of standard sedimentation coefficient of ~8.6S and a heavy form (H) of ~12.5S (Edelstein et al, 1975). In some instances, minor components - a very heavy form (HH) of ~16.6S and a very

light one (LL) of ~5.1S were observed (Gibson et al, 1976). Most likely H looks to be a dimer of L, HH results from the aggregation of H and LL results from the split of L. The values reported for the molecular weight of the 9S(L) form range from 170,000 (Gibson et al, 1976) to 360,000 (Meunier et al, 1972). However, agreement exists between the estimates values by electrophoresis in SDS after crosslinking -260,000 (Biesecker, 1973), 275,000 (Hucho, 1973) and osmometry -270,000 (Mártinez Carrion, 1975). This last value is quite consistent with the 8-9 nm diameter of the particles observed by electron microscopy in purified preparation of receptor from E. electricus (Cartaud et al, 1973) and T. marmorata (Cartaud et al, 1975). These bracelet-like particles have a characteristic electron-dense central pit and a multi-subunit structure (6 or 5 subunits, 3-4 nm in diameter).

Evidence has been accumulated to support the suggestion that the receptor protein in detergent solution is an oligomer made up of only one class of polypeptide chain of 40,000 apparent molecular weight; the exact number of chains per 9S oligomer is not known but 6 or 5 is plausible (according to Hucho, 1973). The significance of the other bands is not known but the one of 43,000 could represent a membrane-bound component.

For more details about chemical properties and kinetic behaviour of the ACh-receptor, see Heidmann and Changeux, 1978.

(ii) Acetylcholinesterase. Before a second impulse can be transmitted at a synapse, the original state of the membrane must be reestablished and ACh must be rapidly eliminated in a refractory phase. In the adrenergic system removal of transmitter is affected by a reuptake system but there is no cholinergic counterpart of this mechanism. It has been calculated that at many synapses, diffusion alone could remove ACh rapidly enough to account for the observed decay of transmitter action (Hubbar, 1972). Nevertheless, hydrolysis by AChE is certainly the predominant method of removal.

The maximal rates of cell firing in Electrophorus are ~1000 impulses/sec (McIlwain and Bachelard, 1971), and 80% of the acetylcholine must be hydrolyzed between impulses. It can then be calculated that the concentration of AChE at the electroplax junction is $6 \times 10^3$ molecules/$\mu^3$ which is higher than the ACh concentration and sufficient to fulfil its function (Wilson, 1971).

Finally, it is quite possible that ACh-receptor and AChE could be part of a supermacromolecule 'in vivo'.

(iii) Relationship of acetylcholinesterase with the membrane. The question of whether AChE is an integral or peripheral membrane protein has already been reviewed earlier in this thesis. Whichever group it falls into, it seems that the active site of the enzyme is oriented towards the outward facing aspect of the membrane at least in the erythrocyte membrane (Coleman, 1973). Silman and Karlin (1967) showed that a membrane preparation of AChE from the electroplax of electric eel presented an anomalous pH dependence. They explained this by saying that the hydrolysis of substrate by

AChE generating $H^+$, caused a pH fall in the unstirred layer around the membrane compared with the bulk solution. Thus, hydrolysis of substrate by the pH-dependent enzyme was decreased. They found that adding buffer to the solution combatted this effect stabilizing the pH. Also it was found that using a poor substrate reduced the pH dependent inhibition by lowering the rate of $H^+$ generations. This anomalous pH dependence is not observed in the solubilized enzyme.

The importance of the membrane to AChE has also been shown by the effects of lipid fluidity in the erythrocyte on the allosteric properties of the enzyme. (Blog et al, 1973; Massa et al, 1975). They showed that when rats were fed corn oil there was an increased fluidity due to the raised levels of unsaturated fatty acids incorporated into the membranes. When lard was fed to the animals, the opposite situation applied because of the high levels of saturated fatty acids. The erythrocyte bound AChE from rats fed on corn oil showed allosteric behaviour whereas those which were fed lard oil did not show this phenomenon. Also they showed that insulin abolished the allosteric behaviour in corn oil fed rats and cortisol induced it in lard fed rats. It was suggested that this was brought about by the decrease and enhancement respectively of membrane fluidity. Farias et al (1975) make the point that it is tempting to stress the importance of the lipid membrane changes on allosteric regulation of AChE in neuronal function. The above observations might suggest certain hydrophobic interactions of AChE with the membrane. (Aloni and Livne, 1974) even though as many workers feel that it is mainly associated with the membrane by electrostatic inter-

actions because the enzyme can be extracted by high salt concentrations from erythrocyte ghost (Mitchell and Hanahan, 1966)

# SECTION II

# METHODS

# SECTION II: METHODS

## A. ASSAYS

### 1. Acetylcholinesterase

#### a. pH-stat:

Assay by pH-stat depends on the continuous and automatic titration of $H^+$ ions liberated by ester hydrolysis (Wilson and Cabib, 1954).

$$CH_3-CO-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3 \overset{+H_2O}{\rightleftharpoons} CH_3-COO^- + H^+ + OH-CH_2-CH_2-\overset{+}{N}(CH_3)_3$$

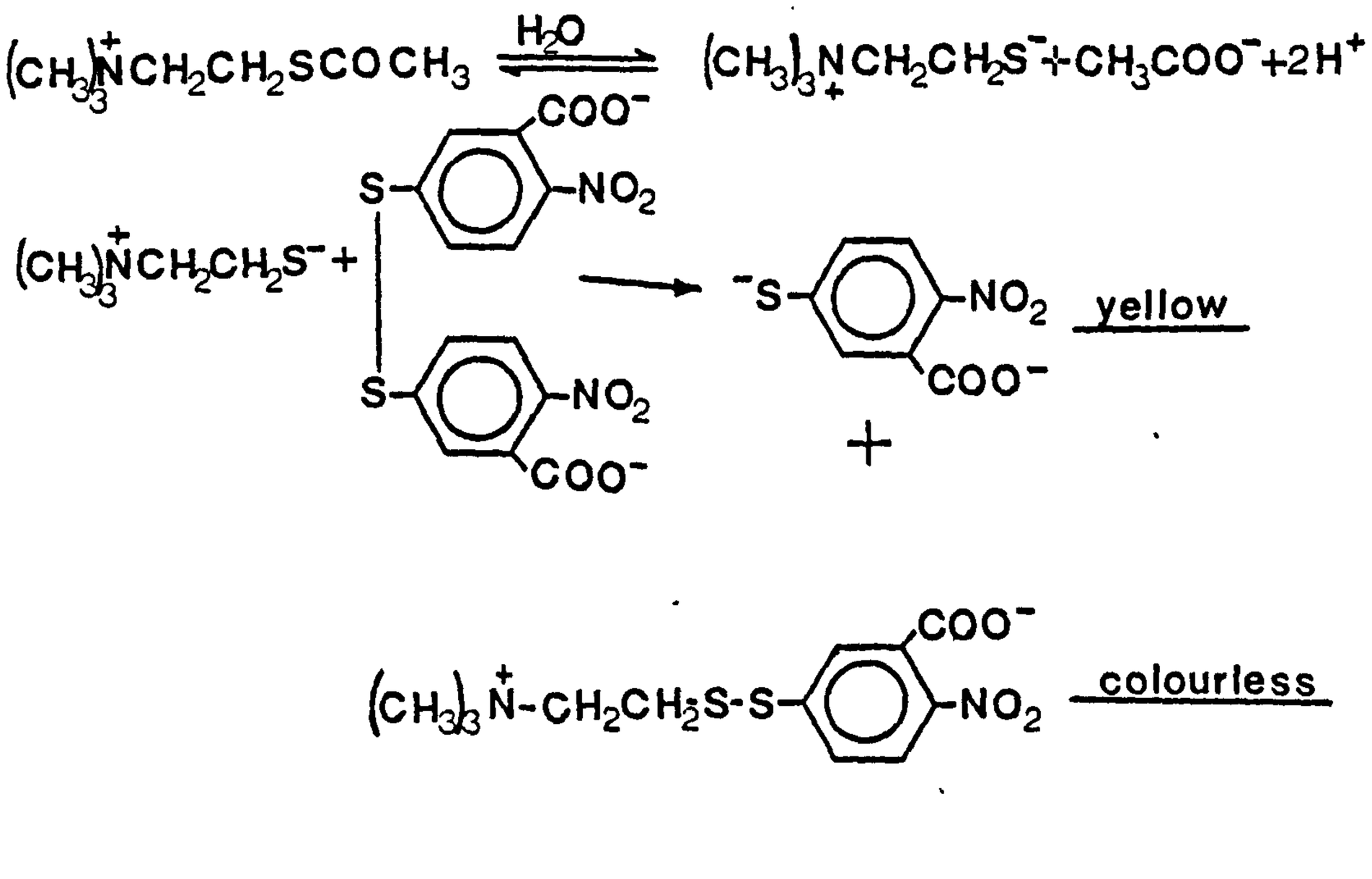
The technique is based upon an earlier manual technique whereby a constant pH was maintained by the addition of sodium hydroxide from a manually operated burette (Glick, 1937). In the modern apparatus a glass-calomel electrode system connected to a pH-meter registers the pH of the reaction medium. This in turn automatically directs the addition of sodium hydroxide from a burette into the reaction vessel to keep the pH at a constant predetermined value. The volume of sodium hydroxide consumed is monitored by a pen recorder. No buffer is necessary in the assay medium although with highly active membrane preparations of enzyme it is advisable to have at least a low concentration of buffer present in order to reduce the effect of a pH gradient at the membrane surface (Silman and Karlin, 1967).

AChE was routinely assayed by adding 0.5 - 1 ml of enzyme sample to 7.2 - 7.7 ml of 0.15 M NaCl + 1.3 m M $MgCl_2$ and measuring the spontaneous release of $H^+$ ions for 5 min. at 37°C and pH 7.6 with sodium hydroxide 20 m M. After this, 0.3 ml of

acetylcholine iodide was added to a final concentration of 1 m M and the enzyme activity measured for at least further 5 min. When kinetic measurements were made at low concentrations of substrate below 5 $\mu$M a second burette containing 20 m M acetylcholine iodide was employed so that a constant substrate was maintained for a sufficiently long time to keep the titration curve rectilinear or only slightly curved. (Jensen-Holm, 1961).

b. Ellman spectrophotometric method

The Ellman method (Ellman et al, 1961) is confined to thioester substrates. The mercaptan formed from the hydrolysed thioester reacts with an oxidising agent, 5,5'-dithiobis- (2-nitro benzoic acid) (DTNB). The DTNB splits into two products one of which 5-thio-2-nitrobenzoate, absorbs at 412 nm.




Brownson and Watts, (1973) reported that the DTNB interacts with AChE causing a marked activation, and so they recommended the use of 2-2' -dithiodipyridine as the oxidizing agent. Augustisson

and Eriksson (1974) however disagree with these findings and advise the continued use of DTNB in the AChE assay.

For routine assay, 50 µl of enzyme was added to 3 ml sodium phosphate buffer (0.1 M. pH8) and incubated at 30°C for 5 min. then 100 µl DTNB (10 m M) was added to the medium followed by 20 µl acetylthiocholine iodide (158.5 m M) to give a final concentration of 1 m M. The increase in absorbance was followed at 412 nm on a Perkin-Elmer SP 124 double beam spectrophotometer.

From the extintion coefficient of the chromophore which is $1.36 \times 10^4$ lit. $mol^{-1}$. $cm^{-1}$. the activity of the enzyme can be thus calculated:

$$\frac{\Delta E \times 1000 \times 3.17}{\text{min.} \times 1.36 \times 10^4 \times 0.05} = \frac{\Delta E}{\text{min}} \times 4.66 \text{ µmol. min}^{-1}\text{. ml}^{-1}$$

The stock DTNB was made up by dissolving 39.6 mg in 10 ml sodium phosphate buffer (0.1 mol/l, pH 7.0) containing 15 mg sodium bicarbonate. The reagent is unstable at more alkaline pH's.

c. Comparison of both methods

The pH-stat is a quite reliable method when the enzyme preparation has an activity higher than 0.15 µmol/min/ml, under standard conditions (37°C and pH 7.6), otherwise it is very difficult to establish the exact value of the activity. However, the method is very useful for thermodynamic and kinetic studies with different substrates and inhibitors because no special requirements are necessary. In contrast the Ellman method is more sensitive and faster but its use is limited to substrates containing thiol groups and

therefore it cannot be used with natural substrates. It is desirable to reserve this method for the measurement of a large number of samples collected from the same experiment but not for comparative individual studies. Throughout the work of this thesis, the pH stat method has been used to measure the activity of preparations derived from solubilization procedures, determination of Michaelis constant and Arrhenius plot experiments. The Ellman method was used to check the activity in all fractions collected from affinity chromatography column, electrophoresis on starch block, and sucrose gradient centrifugation.

When the Ellman method was used, neither inhibition nor activation of enzyme with concentration of DTNB was observed, but a decrease of Km value for Triton solublized enzyme was found (265 μM using pH-stat, acetylcholine as substrate, against 63 μM using Ellman method, acetylthiocholine as substrate. By both methods, the enzyme showed inhibition by excess of substrate. From any of the preparations studied, 1 unit of activity by Ellman method (1 μmol of Acetylthiocholine/min/mg protein) corresponds to 1 units by pH-stat method (in μmol Acetylcholine/min/mg protein).

## 2. Protein Estimation

Protein was accurately determined by the method of Lowry et al (1951) using crystalline bovine serum albumin as standard. However, if Triton X-100 was present, a gelatinous precipitate formed, but this interference could be overcome by centrifuging the precipitate (1000 g. 5min) and incorporating Triton X-100 in the

reagent blank and standars. (Hartree 1972; Chanda-Rajan et al, 1975).

When the amount of protein was high enough,a modified biuret method was used (Plummer,1977).

## B. METHODS OF.SOLUBILIZATION

Wistar rats were beheaded and the brains were removed, washed in ice-water, dried with filter paper and stored at -20°C in the deep freeze until required or at 4°C in toluene containing anhydrous $CaCl_2$ to remove mucins for at least three months. Total membrane-bound AChE was obtained by homogenizing one gram of tissue in 10 ml of sodium phosphate buffer (30 m M. pH 7.0) in a mortar with acid-washed sand, after 1 min., most of the sand was settled and the membrane suspension was centrifuged on an MSE SS65 or SS50 preparative ultracentrifuges using 10 x 10 ml capacity rotors. The criterion of solubility was taken as the enzyme remaining in the supernatant after being centrifuged at 100,000 g for 1 h.

### 1. Triton X-100

The method of Ho and Ellman (1969) was followed with slight modifications. A 10% W/V homogenate of brain cortex was prepared in phosphate buffer (30 m M. pH 7.0) and centrifuged at 100,000 g for 1 h. The supernatant was decanted and subsequently used as "soluble" preparation of enzyme. The pellet was resuspended in the same volume of buffer and Triton X-100 added to a final

concentration of 1%W/V. The mixture was rehomogenized in the cold room (+4°C) and centrifuged at 100,000 g for 1 h. The supernatant was taken as "Triton solubilized" enzyme.

2. Toluene Treatment

The cortices were removed from toluene and and dried on petri-dish until the toluene had evaporated then,finely ground giving the appearance of an acetonic-powder. The homogenization was performed as the above protocol. The fraction solubilized with buffer was taken as "toluene solubilized enzyme" and the corresponding solubilized with Triton-containing buffer as "toluene-Triton" solubilized enzyme.

3. Solubilization in Presence of Antiproteases Agents

The same protocol was used but in this case the extraction buffer contained EGTA (10 m M), benzamidine chlorhydrate (2 m M) N-ethylmaleimide (5 m M), leupeptine (40 µg/ml), pepstatine (20 µg/ml) andbacitracine ( 1 mg/ml) with or without Triton X-100 1% W/V. The supernatants were taken as "antiprotease medium : naturally soluble" and "antiprotease medium Triton solubilized".

4. Autolysis

A 10% homogenate of brain cortex in sodium phosphate buffer (30 mM; pH 7.0) was centrifuged at 100,000 g for 1 h. and the pellet resuspended in the same volume of buffer and incubated at 35°C for 18 h. The suspension was then centrifuged at 100,000 g for 1 h. and the supernatant taken as "autolysis solubilized enzyme".

5. Proteases Digestion

The pellet from the first centrifugation was resuspended in buffer containing trypsin (0.5, 1 or 2 mg/ml), collagenase (0.5, 1 or 2 mg/ml) or papain (50, 100 or 200 µg/ml). The suspensions were incubated at room temperature overnight except in the case of papain when 5 min. appeared to be sufficient. All the mixtures were centrifuged for 1 h. at 100,000 g. The supernatants were taken as "trypsin solubilized", "collagenase solubilized" and "papain solubilized" acetylcholinesterase.

## C. AFFINITY CHROMATOGRAPHY

Synthesis of affinity columns fell into two stages. Firstly, a beaded agarose resin was activated under stringent conditions of pH and temperature. Secondly, an active site ligand specific for AChE was bound to the resin matrix via a spacer arm. For the first column, the ligand and spacer arm were synthesized "in toto" then coupled to the activated agarose. The second column was constructed by consecutively activating the agarose, coupling the spacer arm and then attaching the ligand.

### 1. Materials

Materials for both affinity columns were obtained from the following sources: 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride was purchased from Aldrich Chemical Company Ltd., Wembley, Middlesex. Anhydrous H Br in glacial acetic acid, isobutyl-chloroformate, iodomethane, 9-chloroacridine and

aminopyridine were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Phenol was obtained from Fisons and redistilled before use.

## 2. MAC-agarose Column

### a. Preparation of the ligand (1-methyl-9-($N^{\beta}$-ε-aminocaproyl)-β-aminopropylamino) acridinium bromide hydrobromide

The liquid was synthesised by the method of Dudai and Silman (1974). See Fig. II-1. Phenol (1.28 mol; 120 g) was heated to 70°C and 9-chloroacridine (93.6 m mol; 20g) added to the melt. After all the solid had dissolved 100 ml 1,2-propylenediamine was added to the vigorously stirred mixture and the temperature raised to 120°C. It was critical that this temperature was not exceeded. After 30 min. the product 9-(β-aminopropylamino) acridine (compound I ) was precipitated by pouring the reaction mixture with rapid stirring into 1600 ml of NaOH (0.75 M). This was left over-night to complete the solidification, filtered and washed with NaOH (2M) followed by water. The crude product was dried in vacuo then refluxed in 1100 ml absolute benzene and filtered. The insoluble residue was discarded and the filtrate concentrated to 2000 ml. Then, 50 ml petroleum was added to the filtrate and left at 4°C over night. The crystalline product was filtered and washed with benzene.

yield of 9-(β-aminopropylamino) acridine (compound I) = 9.5 g (39.9%)

m.p. found = 131-139°C

m.p. lit = 131-133°C

The mixed anhydride method of Greenstein and Winitz (1961) was

employed for the next step. N-Benzyloxycarbonyl-Ɛ-amino-caproic acid (30 mmol; 7.9 g) was dissolved in 150 ml dry ethyl acetate in a salt-ice bath at -10°C and stirred vigorously. To this mixture was added triethylamine (30 m mol; 3.05 g) followed by isobutylchloroformate (30 m mol; 4.1 g) and this was left stirring for 20 min at -10°C. The reaction mixture was filtered by suction and the precipitated, triethylamine hydrochloride, washed with 20 ml of dry ethyl acetate. The combined filtrate and washings was returned to the soft-ice bath and a solution of 9-(β-aminopropyl-amino) acridine (25 m mol; 6.3 g) dissolved in dry dimethylforma-mide (100 ml) at -10°C was added. This was left for a further 20 min. at this temperature and then for 12 hours at room temperature. Thin layer chromatography on silica gel in glacial acetic acid in-dicated that there had been total coupling to give compound II. The reaction mixture was evaporated to dryness under reduced pressure and the residue dissolved in 50 ml absolute methanol. Then, 8 ml iodomethane was added to the solution, refluxed for 4 h and left over-night at room temperature. Silica gel thin layer chromatography in ethyl acetate showed that quaternization had gone to completion. The solution was evaporated dry under reduced pressure, extracted twice with 100 ml aliquots of dry ethyl acetate and the product re-crystallized from 80 ml 2-propanol. The solid compound III, [N-(N-benzyloxycarbonyl-Ɛ-aminocaproyl)-(β-aminopropylamino)] acridinium iodide was washed with ice cold 2-propanol followed by ice cold diethyl ether.

Yield compound III = 7.2 g.

m.p. found = 155-156.5°C

m.p. lit = 156-157°C.

The above quaternary compound III (6.4 m mol; 4.1 g) was dissolved in 40 ml anhydrous glacial acetic acid and then 80 ml anhydrous HBr in glacial acetic acid was added. This solution was left for 30 min at room temperature and the product precipitated with dry diethyl ether. The precipitate was triturated with 5 batches of diethyl ether until it solidified. The solid was filtered, washed with diethyl ether and left for 24 h. under reduced pressure over dry NaOH pellets. The resulting crystals of [1-methyl-9-(N$^{\beta}$-$\mathcal{E}$-aminocaproyl)-$\beta$-aminopropylamino] acridinium bromide hydrobromide, MAC, were recrystallized from absolute ethanol.

Yield of MAC = 2.5 g (72%)

m.p. found = 236-240°C

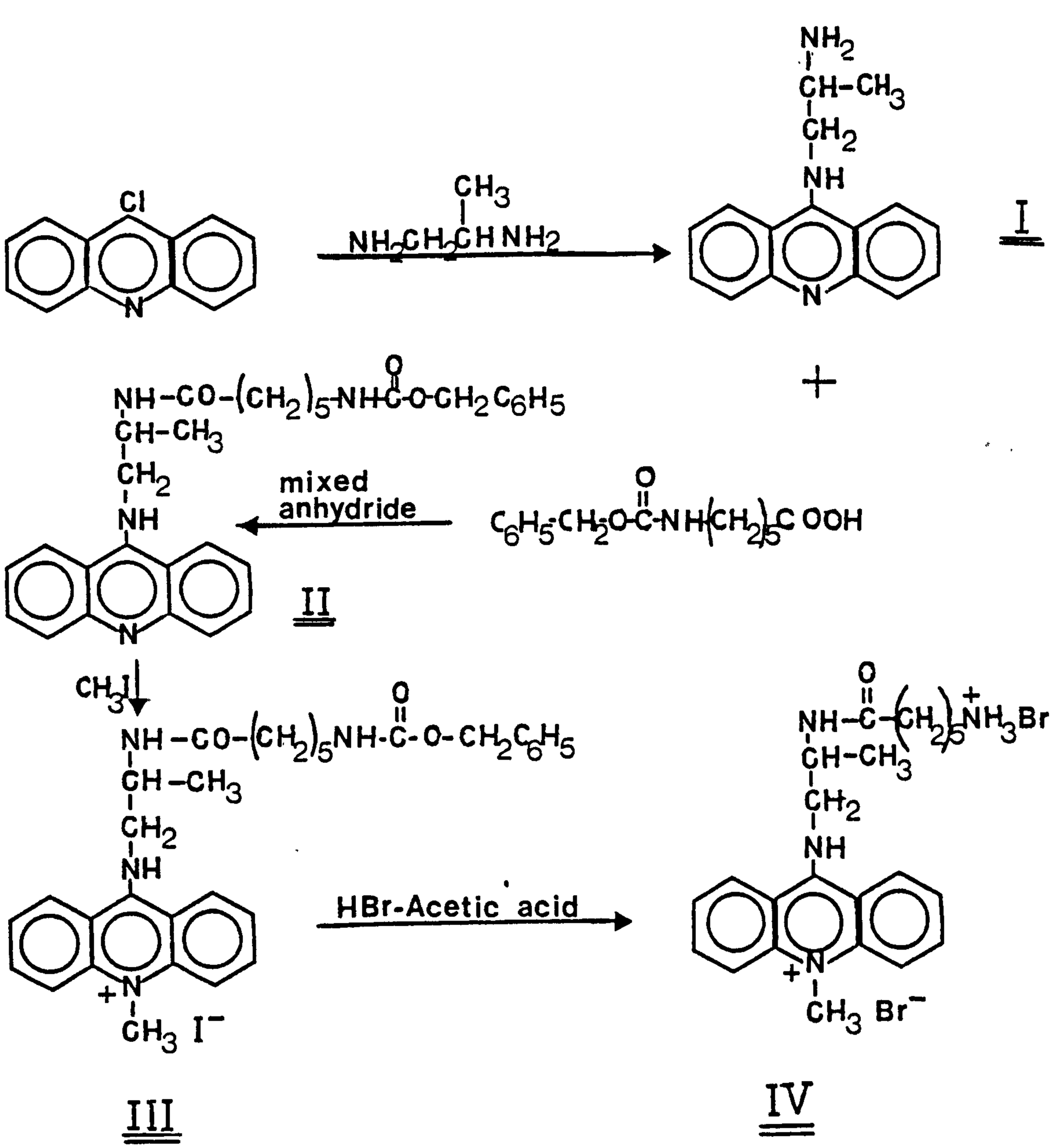
m.p. lit = 240°C with decomposition

b. Preparation of MAC-agarose

The ligand was coupled to the Sepharose 4B by a method based on that of Axen et al (1967) as modified by Blumberg et al (1970). Cyanogen bromide (7 g) was added to 70 ml water and stirred for 10 min. during which time most of it dissolved. A Sepharose 4B slurry was washed with water and 70 ml of this added to the stirred cyanogen bromide-water mixture. The pH was immediately adjusted to 11 with 6M-NaOH and the mixture cooled below 20°C with crushed ice for 8 min. The activated gel was then rapidly washed with 1000 ml water at 4°C on a Buchner funnel and the wet

FIG. II-1

Synthesis of MAC



Sepharose 4B added quickly to a solution of MAC (0.09 mmol, 50 mg) in 30 ml NaH $CO_3$ (O.5M). This mixture was shaken gently (not mechanically stirred) for 16 h at 4°C, then filtered and washed thoroughly with $NaHCO_3$ (0.1M) and water. By spectrophotometric assay of the washings, between 0.5-1.0 µmol MAC were found to be coupled per ml resin.

## 3. MAP-agarose Column

### a. Preparation of the ligand N-methyl-3-aminopyridinium iodide

Aminopyridine (53 mmol; 5g) dissolved in 75 ml acetone was added to iodomethane (292 mmol; 15 ml) and stirred for 18 h. The precipitated N-methyl-3-aminopyridinium iodide (MAP) was filtered and washed with acetone (Fig. II.,2).

Yield MPA = 11.2 g (95%)

m.p. = 120-122°C

m.p.lit = 119-120°C.

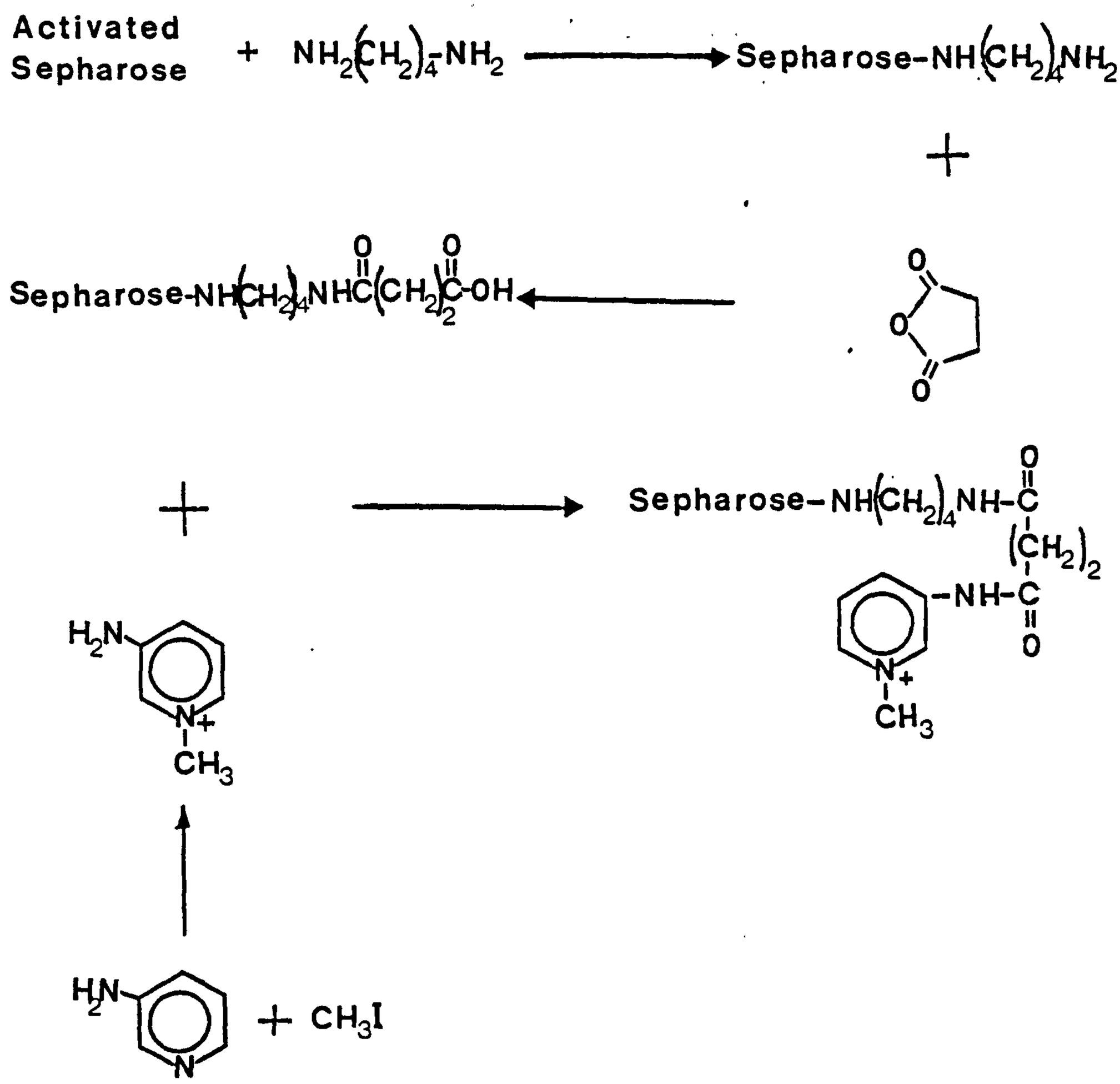
### b. Preparation of MAP-agarose

The affinity resin was built up step-wise by a method based on the procedures used by Berman et al (1971) and Goodkin et al (1974).

100 ml washed Sepharose 2B was suspended in 100 ml water. Cyanogen bromide (30g) was added to the stirred medium and the temperature kept below 20°C with crushed ice and pH at 11.0 with NaOH (4M). After 12 min. the suspension was filtered rapidly under suction with 1000 ml sodium borate buffer (0.1M, pH 9.5) at 5°C.

FIG. II.2

Synthesis of MAP-Agarose



Diaminobutane (198 m mol; 17.4 g) in 100 ml sodium borate buffer (0.1 M; pH 9.5) was immediately added and the mixture shaken gently at 4.0°C overnight.

The excess amine was removed by filtration on a sintered glass funnel and the washed resin dispersed in 100 ml water. Succinic anhydride (100 m mol; 10 g) dissolved in 105 ml water at 4.0°C was added to the resin and the pH raised to 6.0 with NaOH (4M). After the pH had become stable, the suspension was left for 5h at 4°C.

N-methyl-3-aminopyridinium iodide (0.6 m mol; 0.13 g) was added to 6.0 ml succinylated resin in the presence of 1-(3-dimethyl-aminopropyl)-3-ethyl carbodiimide hydrochloride (4.5 m mol; 0.86 g). The reaction mixture was then shaken gently overnight at 4°C and then thoroughly washed before use with elution buffer.

4. Conditions for Elution

The buffer used for elution was sodium phosphate buffer (30 m M, pH 7) 1% in Triton X-100, which was run through two columns connected bottom with top, presenting 12 ml bed volume each. The enzyme was run through the resin at a rate of 30-40 ml/h, after which the column was washed with 5-10 columns volumes of buffer. The enzyme was then eluted with 5 columns volumes of elution buffer containing the AChE inhibitor decamethonium bromide (different concentrations were used) and fractions of 2-5 ml collected. Finally the column was washed with 5 columns volumes of buffer containing NaCl (1 M) followed by guanidine hydrochloride (6 M) and then 50 columns volumes elution buffer. The peak of enzyme active was treated with different

types of cation-exchange resins because the inhibitor was not removed after dialysis even over one week. All the operations were conducted at 4°C.

The above elution programme is similar to the one used by Dudai et al (1972a); Dudai et al (1972b); Berman and Young (1971); Goodkin and Howard (1974) with extensive modifications.

## D. ELECTROPHORESIS

### 1. Polyacrylamide in Rods. Ornstein (1964); Davis (1964).

Stock solutions were prepared as follows:

| Solution A | | Solution B | |
|---|---|---|---|
| 1 M HCl | 48 ml | 1 M HCl | 48 ml |
| Tris | 36.6 g | Tris | 5.98 g |
| TEMED | 0.25 ml | TEMED | 0.46 ml |
| Water to | 100 ml | Water to | 100 ml |
| pH | 8.9 | pH | 6.9 |

Adjust with 1 M HCl

| Solution C | | Solution D | |
|---|---|---|---|
| Acrylamide | 28.0 g | Acrylamide | 10.0 g |
| Bis | 0.735 g | Bis | 2.5 g |
| Water to | 100.0 ml | Water to | 100 ml |

| Solution E | | Solution F | |
|---|---|---|---|
| Riboflavin | 4 mg | Sucrose | 40 g |
| Water to | 100 ml | Water to | 100 ml |

Working Solutions

| Small Pore Solution 1 | Small Pore Solution 2 | |
|---|---|---|
| 1 part A | Ammonium persulphate | 0.14 g |
| 2 parts C | Water to | 100 ml |
| 1 part water | | |

| Large Pore Solution | Stock buffer solution | |
|---|---|---|
| 1 part B | Tris | 6.0 g |
| 2 parts D | Glycine | 28.8 g |
| 1 part E | Water to | 1 litre |
| 4 parts F | pH | 8.3 |
| pH 6.7 | | |

Equal volumes of small pore solutions 1 and 2 were mixed, degassed and aliquots of 0.9 ml added to eight electrophoresis tubes. A water overlay was applied to the surface of the solutions and these were left to polymerise (20-40 min). The water overlays were removed and 0.15 ml degassed large pore solution syringed on top of the gels. The solutions were overlaid with water and the tubes placed in a fluorescent light until polymerisation was complete (20-40 min). A mixture of 150 µl large pore solution and 10-40 µl sample protein was added to each tube and polymerised as described above.

The stock electrophoresis buffer was diluted ten times with water and poured in the anodic and cathodic reservoirs of a Quickfit PAGE apparatus. Eight gels were run per apparatus at a constant current of 2.5 mA per tube for $1\frac{1}{2}$ - 3 h at $4^{\circ}C$ or $40^{\circ}C$. Protein was stained by placing the gels in Coomassie Brilliant Blue stain (0.2%) in methanol: acetic : water in the ratios 5 : 1 : 5 for 24 h. For destaining, the gels were incubated in acetic acid (7% v/ v) for periods long enough to remove the background stain.

AChE was stained by the method of Koelle (1951) as modified by Lewis and Shute (1966). Acetylthiocholine iodide (100 mg) was dissolved in 8 ml water and 14 ml cupric acetate was added dropwise to the stirred solution. This was then centrifuged at 2.000 rpm for 10 min, and the supernatant decanted into a beaker containing glycine (60 mg). Sodium acetate (2 M) was added to the solution to give a final pH between 6.5 and 7.0. Gels were incubated in this stain for 6-24 h and destained in 7% v/v acetic acid.

When the specific anticholinesterase BW 284 C51 dibromide had been used to inhibit the enzyme, the gels were incubated in a solution of the inhibitor (10 µmol/l) for 1 h prior to staining.

2. Polyacrylamide in Slabs

Ready made "Gradipore" polyacrylamide gradient gels (4-24%) were used (Universal Scientific Ltd., London).

Tris-borate-EDTA buffer solutions (pH 8.3) was made up as follows:

| | | |
|---|---|---|
| EDTA | 4.65 g | (2.5 mM) |
| Tris | 53.75 g | (88.74 mM) |
| Boric acid | 25.20 g | (81.51 mM) |

Gels were pre-electrophoresed without samples in a three cell "Gradipore" electrophoresis unit at 100 v until the current had fallen to a steady value between 35-40 mA. Samples (30 µl) were applied to the top of the gels in a plastic spacer which allowed 14 samples to be run per gel. The samples were overlaid with buffer and electrophoresis performed for 24 h at 100 v and room temperature. The buffer was circulated through a heat exchanger immersed in a water bath to keep the temperature stable. Alternatively some electrophoresis were performed at 40°C and 4°C.

Staining of the gels for protein and enzyme was accomplished by removing them from the glass retaining plates and treating them in the same way as polyacrylamide rods.

The method is very sensitive and quite reliable and with this technique it is possible to establish the molecular size of the different forms with reference to a standard curve obtained by plotting

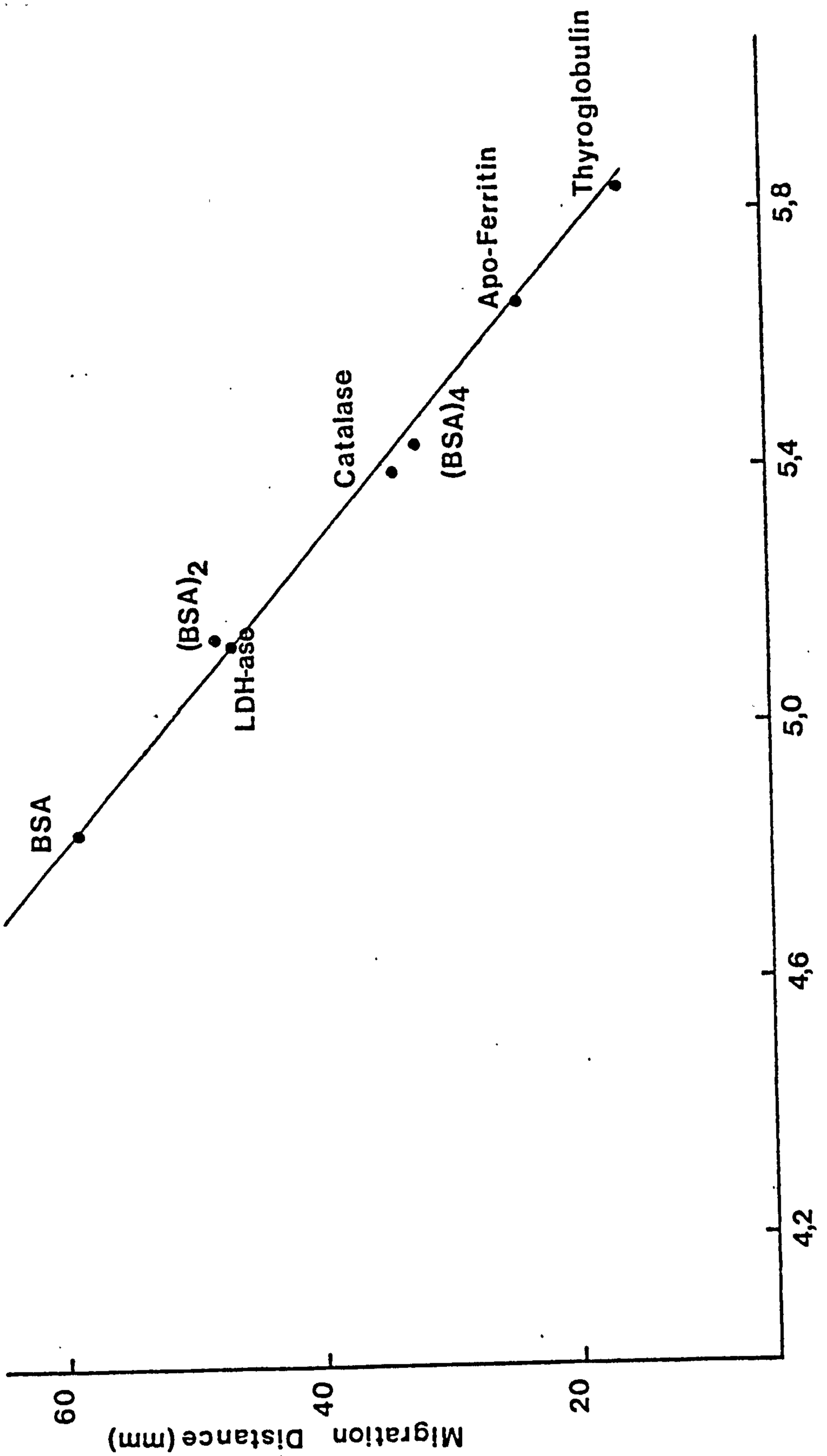



Fig. II.3
Calibration curve for gradient polyacrylamide gels.

migration distance against $\log_{10}$ molecular weight. The proteins employed were thyroglobulin (670,000) apoferritin (450,000), bovine serum albumin (272,000, 136,000, 68,000), catalase (250,000) and lactate dehydrogenase (134,000) (Fig. II.3). However, in order to minimize the differences in two electrophoresis, due to current intensity, buffer concentration and some other factors, it is convenient to run a sample of catalase together with enzyme preparation and check distance for catalase after staining just for enzyme activity (catalase is easily detected by its typical brown spot). It is possible to improve this technique using the above stock solution buffer diluted ten times and under these conditions, the voltage is kept constant at 100 v, and the current drops to 18-20 mA. In this case, it is advisable to apply a low activity of the enzyme (0.3 $\mu$ mol AcCh/min/ml enzyme). In order to avoid excessive background staining due to the detergent, the Triton preparations need to be diluted ten times with buffer.

### 3. SDS-Gel Electrophoresis

This technique was pioneered by Maizel and his collaborators in 1967, and they showed that when proteins are subjected to polyacrylamide gel electrophoresis in the presence of an excess of sodium dodecyl sulphate (SDS), the relative mobilities of the protein solutes were a linear function of the logarithms of their molecular weights. The correctness of this relation was amply confirmed by Dunker and Rueckert (1969), using 24 test proteins and by Weber and Osborn (1969) with 40 test proteins. They showed that the relation was of the type:

$$u = A - B \log M$$

when u is the relative mobility, M is the molecular weight of the solute and A and B are constants for a particular experiment system. An explanation of these results was provided by Reynolds and Tanford (1970), who showed that in the presence of excess of SDS, all proteins, regardless of their native size or shape, bind an equal amount of SDS gel per unit weight. This is virtually constant at 1.4 g SDS/g protein. The binding is apparently mainly hydrophobic and it is completely reversible. The amount of SDS taken up is sufficiently large to swamp the intrinsic charge of the protein, so that to a close approximation the charge per unit mass of the complex is constant. The method is very useful for chain weight determination. Under the appropriate polyacrylamide gel concentration, the molecular weight determinations were within $\pm$10% of the accepted literature values.

Reduction of the protein solutes with 2 mercaptoethanol or dithiothreitol is advisable to obtain minimal values of peptide chain weight but although maximum uptake of SDS is only obtained after complete reduction of disulphide bonds, such reduction does not produce any apparent increase in mobilities, presumably due to a compensatory increase in hydrodynamic volume.

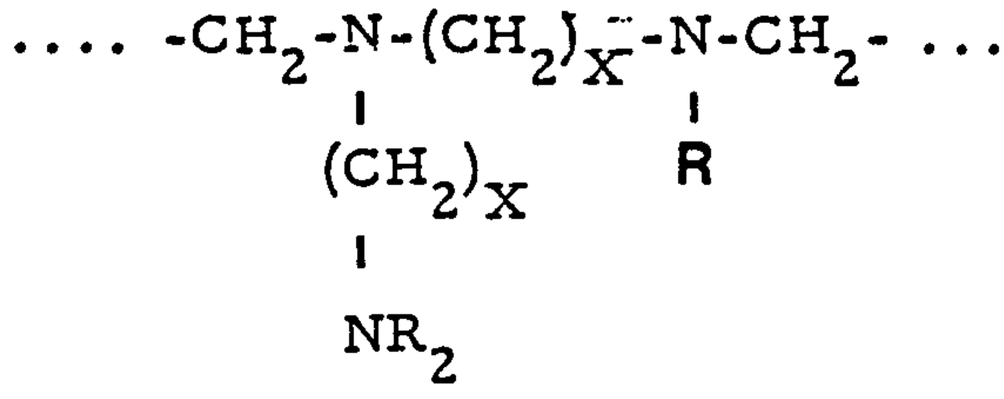
Experimentally, slabs with polyacryalmide gradient were used. The conditions were identical to the normal electrophoresis but this time, the circulating buffer (EDTA, Tris-Boric acid, pH 8.3) contained SDS 0.1% and mercaptoethanol 0.1%. The samples were treated with buffer containing 1% SDS and mercaptoethanol 1%, in the

ratio 1 : 1 and they were heated at 100°C for 3 min. Standard proteins - trypsin inhibitor (21.500); (BSA 68,000); RNA-polymerase (160,000; 39,000) - were treated in the same way. The electrophoresis was performed for 3 h at room temperature. From a plot of logarithm of molecular weight versus relative mobilities of proteins it was possible to calculate the weight of the acetylcholinesterase subunit (Fig. III.31).

## 4. Gel Isoelectric-focusing

Isoelectric focusing differs from the previous methods in that it is an equilibrium method. A pH gradient is set up in the separation channel and solutes migrate from any initial position in the channel to form a sharp Gaussian zone at a pH corresponding to their isoelectric (or more strictly isoionic) point, pI. Equilibrium methods possess two important advantages over the more usual kinetic methods. In the first place, after equilibrium has been attained, the process is time-independent, so that the zone definition does not deteriorate with the further passage of time. Secondly, since solutes migrate from all positions in the channel towards the final equilibrium position, no definite starting zone is required, and the initial solute mixture may indeed occupy the entire volume of the separation channel. Although some isoelectric fractionations of proteins in unstable pH gradients were obtained by Kolin as early as in 1958, the basic problem in realizing the method experimentally lay in the production of a pH gradient sufficiently stable in time to permit extended fractionations. This was achieved in 1962 due to

the important theoretical and experimental investigations of Svensson (Svensson, 1961, 1962 a, b), who showed that a stable pH gradient (a "natural pH gradient") could be set up if a mixture of ampholytes of varying isoelectric points (pI values) was subjected to electrophoresis. Under these conditions, each ampholyte migrates to form a stationary zone in the neighbourhood of its isoelectric point, and since contiguous zones intermix by diffusion, if sufficient ampholytes of appropriate pI values are present, any required pH region can be covered without discontinuity. Svensson also showed that both the electrolytic conductance and the buffering capacity of the isoelectric zone of an empholyte were at a maximum when ($pK_1$ - pI), the difference between the first dissociation constant pK, and the isoelectric point pI of the ampholyte was small. This condition, together with low molecular weight for subsequent removal, and transparency at 280 nm, defined the requisite properties for an ideal ampholytic component of a stable pH gradient. Svensson (1962) used protein hydrolysates as a source of gradient ampholytes and demonstrated the practicability of the method by sharp separations of carboxy haemoglobins A, C and S, in a pH gradient with zone stabilization by means of a density gradient. However, the method had several disadvantages and no general solution of the problem was available until Vesterberg (1969) described the preparation of a wholly synthetic ampholytes by the condensation of various poly-; ethylene-polyamines with $\alpha$, $\beta$ unsaturated carboxylic acid such as acrylic acid and maleic acid to give products of the general type:



where X = 2 or 3, and R = H or $-(CH_2)_X$ - COOH. Since a mixture of reactants was employed, a very complex mixture of product was formed, containing several hundred individual substances, with molecular weights up to about 1000, with some containing up to 10 ionizable groups. Interaction between adjacent ionizable groups produced a wide range of $pK_A$ values, so that the mixture was very suitable for the production of a stationary pH gradient. The large number of individual components with overlapping pK values, provided a relatively uniform conductance throughout the gradient. The mixture had satisfactory transparency at 280 nm. The production of these materials on a commercial scale has been taken up by LKB Producter A.B and they are available under the trade name of "Ampholines".

Experimentally, gel electrofocusing was carried out in 9 cm. long x 7 mm diameter 7% polyacrylamide gel at room temperature. The stocks solutions were prepared as follows:

| Solution A | Solution C | Solution E |
|---|---|---|
| TEMED 0.23 ml | Acrylamide 28.0 g | Ammonium persulphate 0.14 g |
| Water to 100 ml | Bis 0.735 g. g | Water to 100 ml |
| | Water to 100 ml | |
| **Solution B** | **Solution D** | **Solution F** |
| TEMED 1 ml | Acrylamide 10 g | Sucrose 40 g |
| Water to 100 ml | Bis 2.5 g | Water to 100 ml |
| | Water to 100 ml | |

## Working Solutions

| Small Pore Solutions | Large Pore Solution |
|---|---|
| 1 part A | 1 part B |
| 2 parts C | 3 parts D |
| 1 part Water | 1 part E |
| 1/10 part of concentrated Ampholine solution | 1/10 part concentrated Ampholine solution |
| 4 parts of E | 3 parts of F |

Ampholine solution was to cover pH's range 3 - 10.

Aliquots of 1.2 ml were added to eight electrophoresis tubes. When the polymerisation was complete, the water overlays were removed and 0.30 ml large pore solution syringed on top of the gels. The solutions were overlaid with water and when the polymerisation was achieved, a mixture of 100 µls large pore solution and 50 µls sample protein was added to each tube and polymerised as described above.

The stock electrophoresis solutions, ethanolamine (0.4%) and sulphuric acid (0.2%) were poured in the cathodic and anodic reservoirs of a Shandon electrophoresis apparatus with cool water circulation. Eight gels were run per apparatus at a constant voltage of 350 volts for 4 h at room temperature. At the end of that period, most of bromophenol blue used as marker, was removed from the tube. The gels were stained with Coomassie Brilliant Blue for protein.

Determination of the pH gradient along the gel was carried out by slicing the gel into 30 sections which were eluted in small beakers with 2 ml of water for 4 h. The pH was then measured with microelectrodes. A plot of pH against number of section is shown in Fig. III.30.

The above programme is similar to the one used by Wrigley (1968) with some modifications.

## 5. Starch Block Electrophoresis

This method, though too laborious as a routine analytical tool, has its use as a semipreparative procedure. Potato starch was obtained from British Drug House, Poole, Dorset. About 500 g of starch was washed twice with distilled water and twice with buffer (sodium phosphate buffers of various ionic strength and pH were used) After the final wash, the starch was allowed to settle for about 2 h, the excess of buffer removed by decantation and the starch pressed into a block with the aid of perpex formers, 19 x 12 x 1 cm or 30 x 10 x 1 cm. Excess of liquid was removed from the block by blotting with Whatman 3 MM paper. Wicks consisting of several layers of muslin saturated with buffer solution connected the block with the buffer in the electrode tanks. Anode and cathode tanks were each divided in two sections, connected by cotton wool plugs. This arrangement prevented pH changes around the electrodes affecting the buffer in contact with block. The block was pre-electrophoresed at 6-8 v/cm for 1 h at 4$^{o}$C to allow the system to equilibrate. The enzyme sample containing 5-10 mg protein was applied to a laterally cut groove, 0.5 cm wide and one-third of the distance along the block from the cathode. A mixture of haemoglobin, bovine serum albumin and bromophenol blue was applied as markers to a cavity in line with the groove. The cavity and groove were filled with starch and electrophoresis conducted at 6-8 volt/cm for dif-

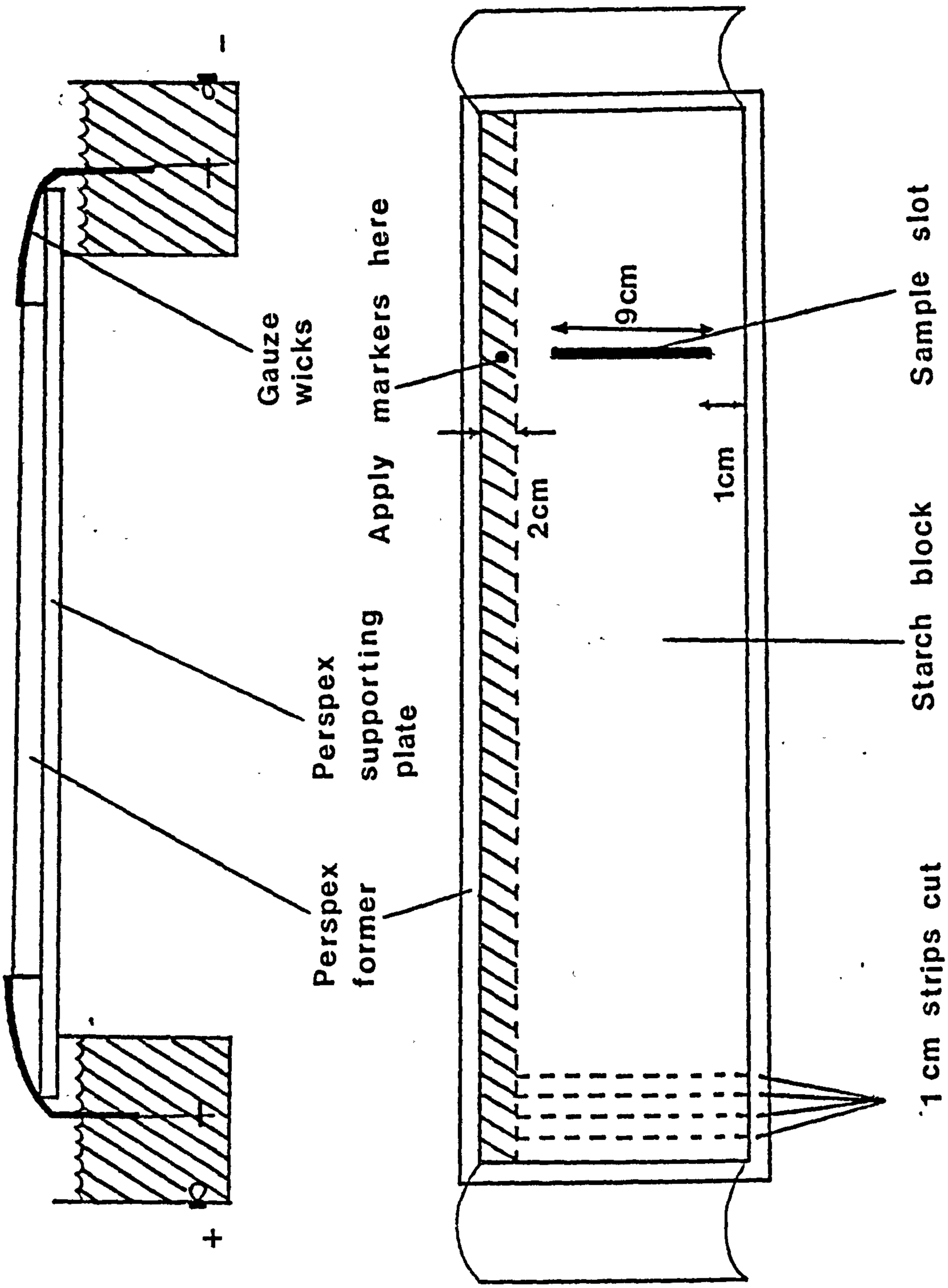

Gauze wicks
Perspex supporting plate
Perspex former
Apply markers here
2cm
9cm
1cm
Sample slot
Starch block
1 cm strips cut transversely
Starch block electrophoresis. (Plummer, 1977)
+
-

Fig. II.4

ferent periods of time, depending on buffer used in each experiment (Fig. II-4).

The final positions of markers were noted and the longitudinal section containing them discarded. The remainder of the block was cut transversely into strips of 0.5 cm width between the markers and 1 cm width either side of the markers. Each portion of starch was eluted twice through a sintered glass funnel with 2 ml aliquots of buffer. The AChE was assayed by the Ellman method and the electrophoresis patterns presented as histograms showing total activity in each strip cut from the block

## E. DENSITY GRADIENT CENTRIFUGATION

In order to determine the sedimentation coefficient and approximate molecular weight of AChE, sucrose gradients were used in the swing-out rotors of a preparative ultracentrifuge. This was possible by determining the ratio of mobilities between AChE and a standard well characterized protein (Martin and Ames, 1961)

### 1. Preparation of Gradients

Sucrose gradients were prepared in 5 ml capacity polycarbonate tubes. The sugar was added in equal layers of diminishing concentration. The bottom layer was 60% w/v and on the top of this were pipetted seven layers of equal volumes (0.5 ml) of buffered sucrose, with or without Triton, starting with 20% w/v diminishing finally to 5% w/v. Sometime the 5% layer was substituted by the corresponding volume of enzyme preparation made up to that sucrose

concentration. The tube was then rotated gently back and forth around its longitudinal axis to disperse the interfaces and thus obtain a homogeneous linear gradient. Gradients were stored at $4^{o}C$ for 5 h before use.

## 2. Centrifugation

The standard protein, bovine catalase, which was assumed to have a sedimentation coefficient of 11.4S and mol. wt. 240,000 (Sumner and Gralen, 1938), was mixed with the enzyme sample and layered by pipette on to the gradients. It was essential that not more than 50 mg protein was applied to each gradient as overloading causes loss of Gaussian shape of the migrating protein zones (Steensgaard et al, 1975).

After spinning ~~for~~ at 100,000 g ~~for~~ 17 h, the tubes were fractionated and aliquots removed with an MSE tube piercer. Fractions of 3 drops were taken from the tube and then assayed for AChE by the Ellman method. Catalase was assayed by following the decrease in absorbance at 420 nm of a mixture containing 3 ml sodium phosphate buffer (10 mmol/l, pH 7.5), 20 µl hydrogen peroxide (0.9 M) and 20 µl enzyme sample. Activities were calculated in terms of change in absorbance/min. The ratio 'R' was determined experimentally according to Martin and Ames (1961):

$$R = \frac{\text{distance travelled from meniscus by unknown}}{\text{distance travelled from meniscus by standard}}$$

As molecules move at an almost uniform rate:

$$R = \frac{S^{0.725}_{20.W}\ \text{unknown}}{S^{0.725}_{20.W}\ \text{standard}}$$

where $S^{0.725}_{20.W}$ = sedimentation constant extrapolated to the standard state taken as that of water and partial specific volume 0.725 $cm^3/g$. As most proteins have partial specific volumes in the range 0.70-0.75 $cm^3/g$, the above assumptions should result in less than 3% error in the estimation of $S_{20.W}$.

So, for molecules of the same partial specific volume:

$$R = \frac{S_{20.W}\ \text{unknown}}{S_{20.W}\ \text{standard}}$$

A crude estimation of mol. wt. can thus be obtained:

$$\frac{S_1}{S_2} = \left( \frac{\text{Mol. wt}_1}{\text{Mol. wt}_2} \right)^{\frac{2}{3}}$$

since for most proteins $S_1/S_2$ is equal to R. (Schachman, 1959). In order to test the linearity of the gradients, some centrifugations were performed in the absence of samples. The sucrose concentrations were then determined by refractometer and plotted graphically against fraction number.

Also, the efficacy of this technique was tested by running three standard proteins on gradients and comparing their sedimentation coefficients with those found by other techniques. The proteins used were yeast alcohol dehydrogenase, egg white muramidase and beef liver catalase. Assays were performed according to Martin and Ames (1961). Sedimentation values were assumed to be those quoted by Martin and Ames: catalase, 11.4S; muramidase 2.1S; A.D.H. 7.6S. Fig. III.16).

# SECTION III

# RESULTS

# A. SOLUBILIZATION OF ACETYLCHOLINESTERASE FROM RAT BRAIN

## 1. Rat Brain Stored in Toluene

In these experiments brains stored in toluene for three to six months at 4°C were used. At the end of that time the brains were removed from the toluene and placed in a Petri dish in the fume chamber until the residual toluene had evaporated. The acetylcholinesterase was then extracted as follows:

### a. Extraction with dilute buffer

The enzyme was considered to be soluble if it remained in the supernatant after centrifugation at 100,000 g. for 1 h. Extraction of a 10% w/v homogenate prepared from dry toluene powder of brain with 0.03M sodium phosphate buffer (pH 7.0) showed that 15-20% was soluble. The specific activity of this preparation was 0.052 μmoles of acetylcholine hydrolyzed per minute, per mg of protein. In Table III.1. values for activity/gr of dry toluene powder and activity/gr of wet weight are given for comparison. This was called 'soluble toluene-brain' fraction'. The remainder of activity (80-85%) was sedimented after centrifuging for 1 h at 100,000 g and was therefore considered to be bound to a particulate fraction (McIntosh, 1973).

### b. Detergent treatment

When the pellet resulting from the first centrifugation was resuspended in the same volume of buffer phosphate containing

## TABLE III.1

### Solubilization of toluene stored rat brain

| Fraction | Yield of activity | Yield of proteins | Specific activity | a/g toluene powder | a/gr wet weight |
|---|---|---|---|---|---|
| 10% homogenate in dilute buffer | 100 | 100 | 0.152 | 31.8 ± 5.6 | 6.5 ± 1.1 |
| 100,000 x g supernatant | 17 | 49 | 0.052 | 5.4 ± 0.8 | 1 1 ± 0.1 |
| Pellet resuspended in buffer-Triton (1% w/v) - 100.000g. Supernatant | 58 | 24 | 0.340 | 18.4 ± 6.0 | 3.75 ± 1.0 |

Average of six different experiments.

Specific activity expressed in μmol AcCh/min/mg protein at 37°C pH 7.6.

Triton X-100 (1% w/v) between 55 and 60% of the enzyme bound to membrane was solubilized. The specific activity in this case reached a value of 0.340 µmol AcCh/min/mg protein (Table III.1) This was referred to as the 'Triton Toluene-brain solubilized' fraction (Fig. III.2a).

## 2. Frozen Rat Brain

In most of these experiments, rat brains frozen for different periods of time were used. When fresh rat brain was tested no appreciable difference in activity nor proteins was observed because all the values were fitted properly into the standard deviation for the total set.

### a. Soluble fraction

Extraction of a 10% homogenate of brain with 0.03M sodium phosphate buffer (pH 7.0) showed that 10 to 15% of the AChE was soluble; this fraction was considered as "naturally soluble enzyme". The specific activity was 0.036 µmol AcCh. $min^{-1}$. mg $protein^{-1}$ (Table III.2). The remainder of activity (85-90%) was assumed to be bound to the particulate fraction. However a second extraction of the pellet obtained from the first centrifugation yielded another 4-5% of soluble enzyme but a third one brought into solution only 2-3% (Fig. III.1).

### b. Autolysis

Incubation of the pellet resulting from the first centrifugation

## TABLE III. 2

### Solubilization of Frozen Rat Brain

| Fraction | Yield of activity | Yield of protein | Specific activity | a/gr wet weight |
|---|---|---|---|---|
| 10% homogenate in dilute buffer | 100 | 100 | 0.060 | 12.5 ± 2.4 |
| 100,000 xg supernatant | 13 | 24 | 0.036 | 1.7 ± 0.2 |
| pellet resuspended in buffer containing Triton X-100 1% 100,000 g supernatant. | 105 | 20 | 0.331 | 13.3 ± 4.8 |

Average of six different experiments.

Specific activity expressed in μmol AcCh/min/mg protein at 37°C, pH 7.6.

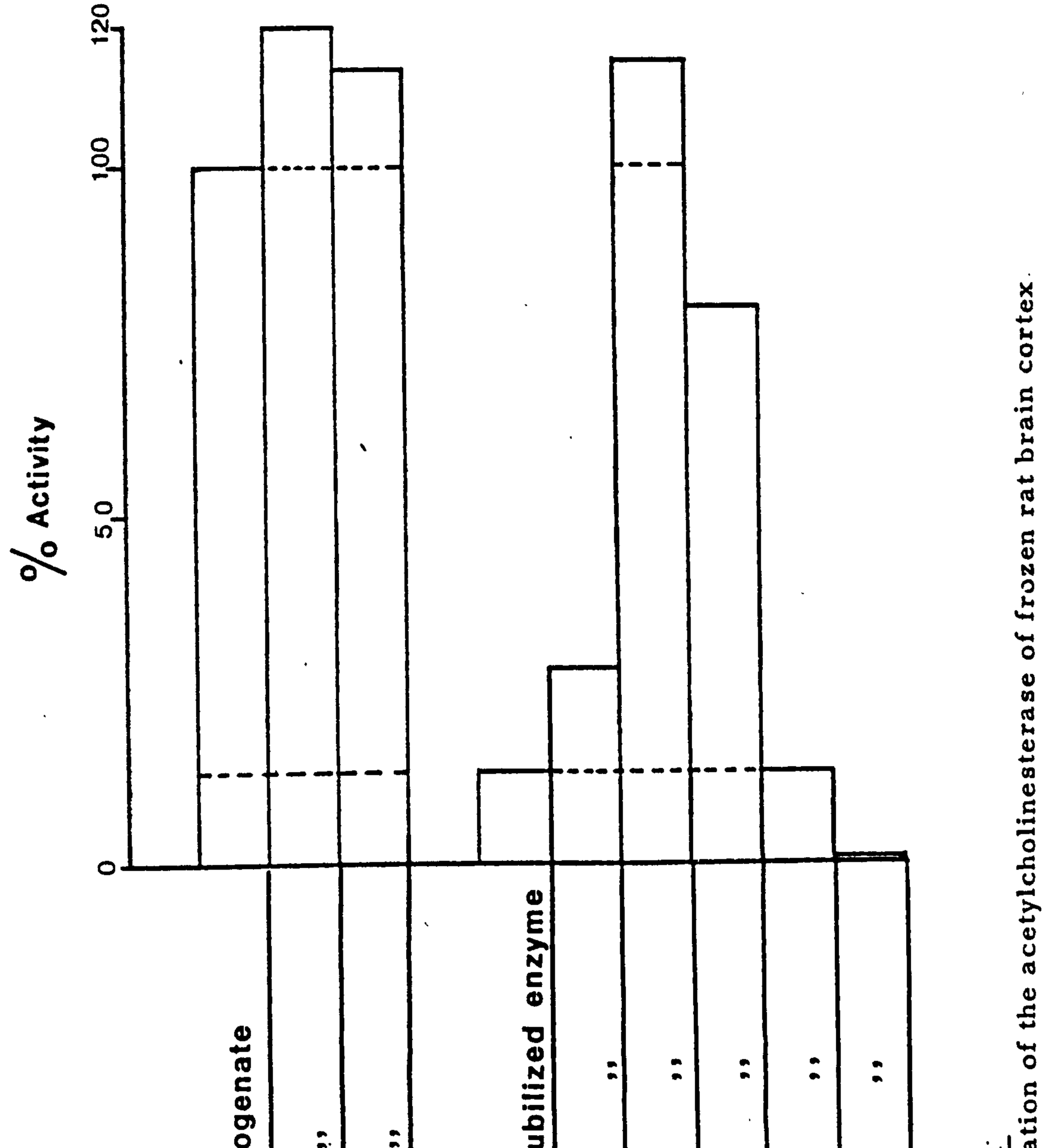



Fig. III.1
Solubilization of the acetylcholinesterase of frozen rat brain cortex.

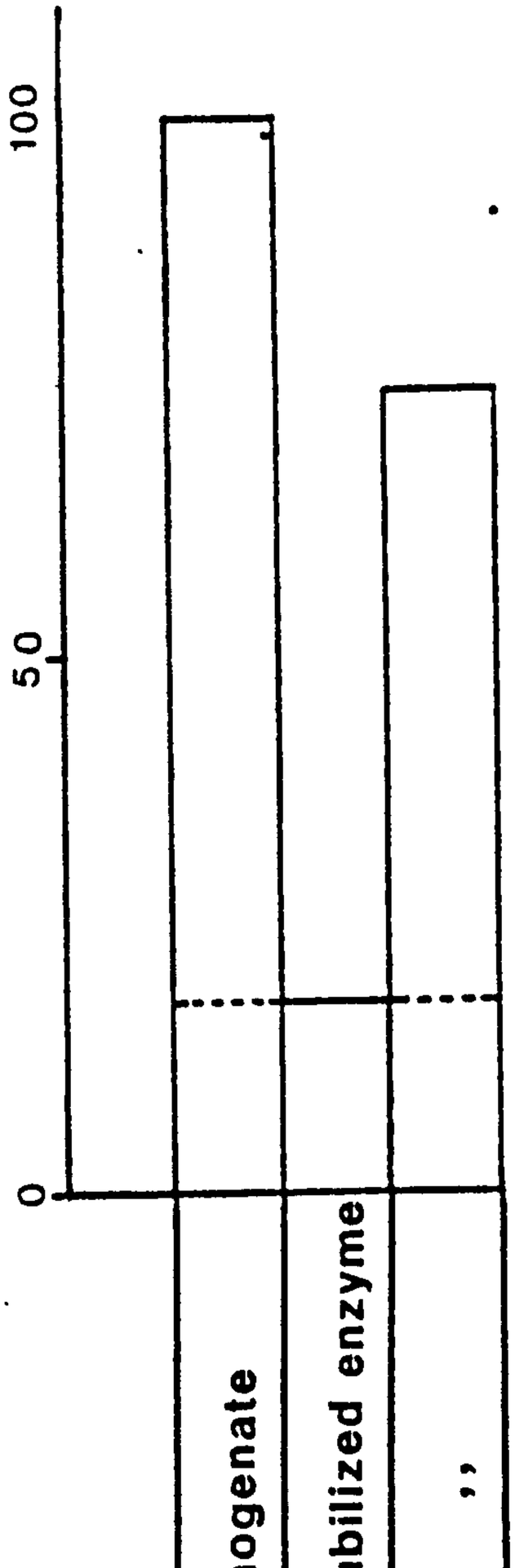



Fig. III.2
Solubilization of acetylcholinesterase from toluene-stored rat brain (a) or from frozen rat brain in the presence of antiproteases agents (b).

for 16h at 35°C yielded over 15-20% of soluble acetylcholinesterase and no change in the activity of the suspension before spining was observed. This is referred as 'Autolytic fraction' (Fig. III.1).

c. Solubilization with Triton X-100

When the pellet from the first centrifugation was resuspended in its volume of dilute buffer containing Triton X-100 (1% w/v) up to 95-100% of the activity bound to membrane was solubilized, and still over 10-15% of activity was found in the pellet suspension. It is interesting to note that the treatment of the 10% homogenate with triton X-100 (1% w/v) produced an activation of 20-25% (Table III.3), with respect to the suspension without Triton X-100. The enzyme solubilized by this procedure was referred as "Triton solubilized enzyme", and its specific activity value was 0.331 µmol AcCh /min/ mg protein (Table III.2) (Fig. III.1).

d. The effect of proteases inhibitors

When the 10% homogenate of fresh rat brain was prepared in buffer containing the antiproteases inhibitors (see Section II, Methods), denaturation in the range of 20-25% was detected. In this case, the fraction corresponding to naturally soluble enzyme represented only 3-4% and in that of Triton 75-80% (Fig. III.2b).

e. Incubation with proteolytic enzymes

In these experiments the effect of three different endopeptidases on solubilization of rat brain acetylcholinesterase was considered. The pellet from centrifugation of a 10% homogenate

## TABLE III.3

### Effect of Triton X-100 on Membrane Suspension

| Fraction | Yield of act. | Yield of protein | Specific act. | a/gr wet weight |
|---|---|---|---|---|
| 10% homogenate in buffer containing Triton X-100 1% w/v | 120 | 120 | 0.060 | 15.0 ± 1.4 |
| 100,000 xg supernatant | 115 | 32 | 0.185 | 14.5 ± 3.2 |

Average of three different experiments.

Specific activity expressed in μmol AcCh/min/mg protein at 37°C, pH 7.6

was incubated with the corresponding amount of protease long enough to effect a change in total activity or in solubilized enzyme after centrifugation.

i. Trypsin. When the pellet was resuspended in buffer containing 1.2 or 5 mg of trypsin per ml and incubated at room temperature or at 35°C for 15h, it was found that in all cases the total activity remained constant and there was no solubilization of AChE bound to membrane when the samples were compared with their respective controls (Fig. III.1).

ii. Collagenase. The above programme was repeated and in this case up to 50% of the total activity was lost after incubation of the pellet with collagenase,5mg/ml,for 15 h at 35°C. Indeed there was no solubilization, even the value corresponding to autolysis was lost. If the incubation was done with lower concentrations of collagenase and for shorter times at room temperature neither denaturation nor solubilization was found (Fig. III.1).

iii. Papain. In this case the pellet from the first centrifugation was resuspended in buffer containing 5, 10 and 20 µg of papain suspension and incubated for 15 h at room temperature. In all cases, there was a small loss of activity but a fairly high amount of enzyme, in fact between 55 and 60% of the particulate fraction, was solubilized. It was decided to reduce the incubation time to 3.2 and 1 hour and no change in the yield of solubilization was seen. Finally the pellet was homogenized in buffer containing 10 µg of papain suspension/ml and after stirring the suspension for 5 min in cold room and centrifugation at 100,000g per 1 h, 65-70% of the activity

## TABLE III. 4

### Effect of Papain on Frozen Rat Brain Solubilization

| Fraction | Yield of activity | Yield of protein | Specific activity | a/gr wet weight ± S.D |
|---|---|---|---|---|
| 10% homogenate with dilute buffer | 100 | 100 | 0.060 | 12.5 ± 2.4 |
| 100,000 xg supernatant | 13 | 24 | 0.036 | 1.7 ± 0.18 |
| Pellet resuspended in buffer containing 10 µg/ml papain suspension. 100.000 g supernatant | 63 | 9 | 0.446 | 7.8 ± 0.3 |

Average of three different experiments.

Specific activity expressed in µmol AcCh/min/mg protein at 37°C, pH 7.6.

## TABLE III. 5

### Effect of Papain on Membrane Suspension

| Fraction | Yield of activity | Yield of protein | Specific activity | a/g wet weight ±S.D. |
|---|---|---|---|---|
| Homogenate 10% with papain (10 µg/ml) | 114 | 100 | 0.062 | 11.1 ± 1.8 |
| 100,000 xg supernatant | 80 | 36 | 0.136 | 10.0 ± 0.9 |

Average of three different experiments.

Specific activity expressed in µmol of AcCh hydrolyzed/min/mg protein at 37°C, pH 7.6

from the pellet was solubilized. By this procedure denaturation of the enzyme bound to membrane was prevented. The specific activity for this 'papain solubilized fraction' was 0.446 μmol AcCh/min/mg protein (Table III.4). It was also found that the treatment of brain homogenate with papain suspension (10 μg/ml) produced an activation of 10-15% (Table III.5).

## B. PURIFICATION BY AFFINITY CHROMATOGRAPHY

### 1. MAC-Agarose Column

One hundred ml of Triton solubilized enzyme with a total activity of 1.724 μmol of acetylthiocholine hydrolyzed per minute and 300 mg of protein (specific activity 5.74) was passed through the column previously equilibrated with sodium phosphate buffer (0.03M, pH7) containing Triton X-100 (1% w/v). Fractions of 4 ml were collected and after 10 tubes the enzyme came out from the column in such a way that only 8% of total activity and 9% of protein became bound to the column. The column was prepared twice and after identical results this procedure was abandoned.

### 2. MAP-Agarose Column

Fifty ml of Triton solubilized enzyme with a total activity of and proteins 862 μmol of acetylthiocholine hydrolyzed per minute and 150 mg of protein (specific activity 5.74) were passed through the column which had been equilibrated with phosphate buffer (pH7, 0.03M) containing Triton X-100 (1% w/v), and as previously the enzyme came out from the column, but this time 90% of the total activity and 80% of protein were bound to the column. When the eluate gave a zero reading for protein, the competitive inhibitor, decamethonium bromide (10mM in buffer, 100 ml) was applied to the column and only 1% of total activity was collected after this treatment. Then, 25 ml of elution buffer containing decamethonium bromide (100 mM) was applied and in this case 7% of the total activity was eluted from the

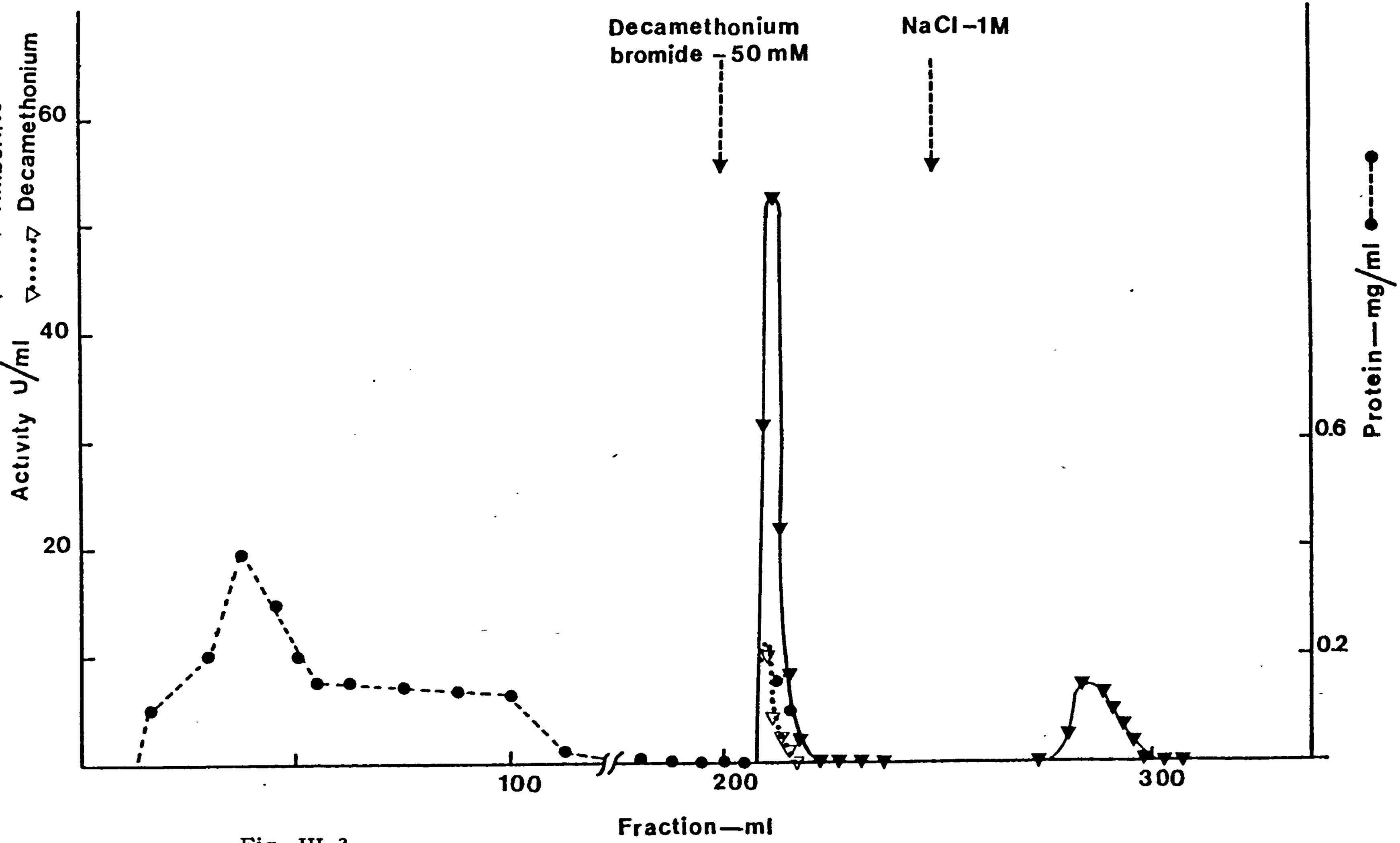



Fig. III.3
Elution profile of AChE from MAC-Agarose Column.

column. The peak of activity was dialysed against 10 liters of buffer for 72 h, after this treatment the activity was not increased and therefore it was considered that the inhibitor remained bound to the enzyme. The specific activity for that fraction was 104 µmol of acetylthiocholine per min and per mg of protein, representing an 18 fold increase compared with the Tritonsolubilized preparation and 130 fold against the crude homogenate (Fig. III.3).

a. Removal of the inhibitor from decamethonium-enzyme complex

The effect of four cation-exchanger resins, CM-Sephadex, CM-cellulose, Dowex 50 x 4 and Amberlite CG-120, was checked on stability of enzyme and on ability to remove the inhibitor from the decamethonium-enzyme complex. The four resins were resuspended in buffer and they were added to the enzyme solubilized with Triton. The enzyme was inactivated completely by Dowex 50 x 4 but there was no inactivation with the other resins. When the other three resins were added to the enzyme which had been completely inhibited with decamethonium bromide (final concentration 100 mM) CM-sephadex and CM-cellulose proved not to have the ability to separate the enzyme from the complex but in the case of Amberlite CG-120 it was shown to be very efficient at releasing the enzyme, and over 80% of the enzyme preparation, completely inhibited by decamethonium, could be collected after this treatment. Therefore, a new preparation of enzyme solubilized by Triton was passed through the column and, as before, 95% of total activity was bound to the column and 75% of the protein.

The enzyme was eluted with buffer containing decamethonium bromide, 50mM and again 6% of the total activity was collected but this time the fractions containing the enzyme were treated with amberlite CG-120, and after that the recovery increased to nearly 70% and the specific activity for the purest fraction was 205 μmol of acetylthiocholine hydrolyzed per minute and per mg of protein, meaning a purification factor of 40 fold against the triton solubilized preparation and 270 folds with respect to the crude homogenate. When NaCl 1M in buffer was passed through the column, an additional 1% of the activity could be recovered but this fraction was highly contaminated with other proteins.

b. Optimum decamethonium concentration for enzyme elution

Triton solubilized enzyme preparation was passed through the column as before, but this time, the column was eluted with buffer containing decamethonium bromide 10mM. No activity was detected even after amberlite treatment. After that, 25mM concentration was tried and in this case, with the amberlite treatment, between 30-35% of the total activity applied to the column was recovered with a specific activity of 16 μmol of acetylthiocholine/min/mg of protein. Therefore, it seems that the higher is the inhibitor concentration - up to 50 mM - the higher is the recovery of the eluted enzyme. In all cases, whatever the inhibitor concentration used with or without amberlite treatment, the preparation of partially purified enzyme was completely inactivated after a week stored in deep-freeze (-20°C).

## C. STABILITY OF ENZYME PREPARATIONS

### 1. Samples Stored in Deep-Freeze

Enzyme preparations obtained from frozen or toluene treated rat brain were shown to be very stable at -20°C. The activity remained constant after 5 or 6 months of storage but after one month turbidity in all preparations was seen. When those preparations were centrifuged at 100,000 g for 1 h a pellet rich in lipid appearance was obtained. That pellet was active, representing 25%, 11%, 11% and 3% for "naturally soluble", "Triton solubilized" "toluene-buffer solubilized" and "toluene-Triton solubilized" enzyme preparations.

### 2. Incubation at 37°C

Those four preparations were shown to be fairly stable against heating because when they were incubated at 37°C for 24 h there was a slight activation in Triton solubilized (15-17%) and "Triton-toluene solubilized" preparations (10-12%). However, incubation of the corresponding samples solubilized by dilute buffer showed some inactivation; 10% for naturally soluble enzyme and 30-35% for buffer-toluene preparation. A considerable increase of turbidity in naturally soluble and buffer-toluene samples was also observed.

### 3. Effect of Cations on Stability of Triton Solubilized Enzyme

#### a. Untreated enzyme

NaCl, KCl, $MgCl_2$, $CaCl_2$ at a final concentration of 0.2M were added to a sample of enzyme or dialyzed enzyme against Tris-

HCl buffer pH 8 0.1M before storage in the deep-freeze (-18°C) and the activity was checked by the Ellman method every 24 h for a week. The results showed that there was no inactivation of enzyme except in the case of NaCl. The addition of NaCl, at a final concentration of 0.2M, to a sample of triton-solubilized enzyme,decreased the activity by about 50% after 3 days of storage and after that no further inactivation was observed (Fig. III.4a).

b. Enzyme treated with Amberlite CG-120

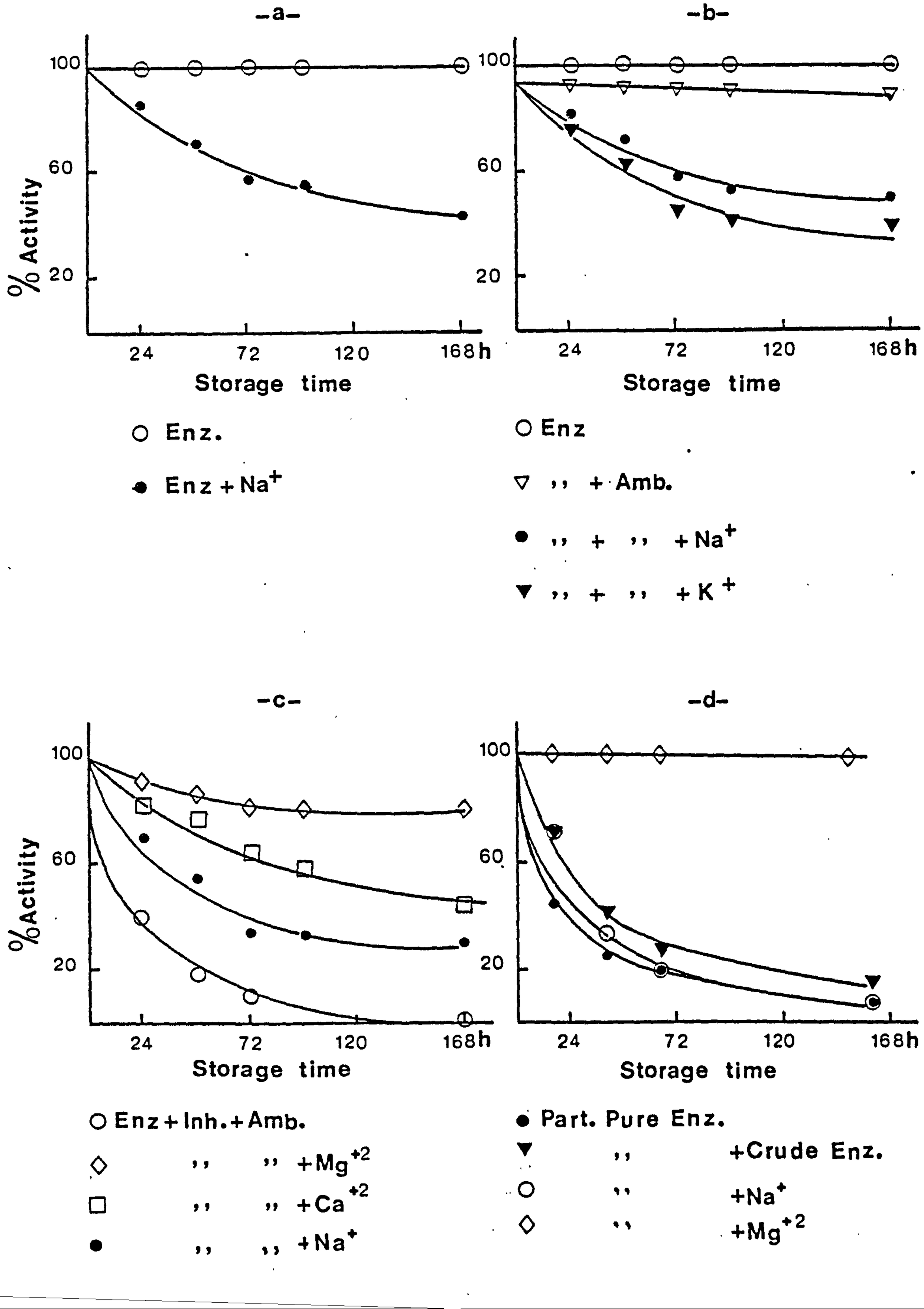
A similar experiment as previously described was carried out but now the enzyme was treated with amberlite. In this case a small inactivation due to this treatment was observed (10%) but after that, the activity remained unchanged. Identical results occurred in samples containing $MgCl_2$, $CaCl_2$, acetylcholine chloride (150 $\mu$M) and egg yolk lecithin (5 mg/ml), but when KCl or NaCl was added the activity dropped by around 50% after 3 days of storage (Fig. III.4b).

c. Decamethonium and Amberlite treatment

The above procedure was followed but this time the enzyme was inactivated with decamethonium bromide (50 mM) and reactivated with amberlite CG-120. It can be seen in Fig. III.4c just how the enzyme is rapidly inactivated. The same behaviour was seen with preparations containing 50 $\mu$l/ml of crude enzyme, lecithin (5 mg/ml), phosphate buffer 0.5M and acetylcholine chloride (150 $\mu$M). The inactivation was also very rapid in preparations containing NaCl or KCl. 0.2M or 0.8M, after 4 days almost 80% of the activity was lost. However, if the enzyme contained $CaCl_2$ the inactivation was retarded

Fig. III.4

Stability of Triton solubilized enzyme on storage in the deep-freeze.



(40% of inactivation after 4 days) but samples containing $MgCl_2$ were shownto be inactivated very slowly and after 3 days only 20% of the initial activity was lost and remained practically constant thereafter.

## 4. Effect of Cations on Stability of Partially Purified Enzyme

A similar experiment to the previous one was performed using the partially purified enzyme by affinity chromatography. When the sample was stored frozen, the same results as with the crude enzyme were obtained (Fig. III.4d). However, samples stored in the cold room (+4°C) did not show that behaviour and after 15 days, just the preparation containing crude enzyme (4 parts of purified enzyme and 1 part of crude preparation) was inactivated by about 40%.

## D. PROPERTIES OF THE ENZYME PREPARATIONS

### 1. Michaelis Constants and Optimum of Substrate

The pH-stat method was used to determine the Km values and the optimum concentration of substrate. The temperature was kept constant at 37°C and the pH reached a value of 7.6 before starting the enzymic reaction. The substrate concentration ranged from 20 to 0.17mM. The preparations studied were the following:

#### a. Membrane preparation from frozen and toluene-stored rat brain

These two preparations were quite similar on the basis of inhibition by excess of substrate. Both of them showed an optimum at at 2mM but the degree of inhibition was slightly higher for the enzyme from toluene-stored than for frozen brain. At 20mM acetylcholine concentration inhibition of 25 and 35% respectively with respect to 2 mM concentration were found. The Km values were 80 μM and 75μM for frozen and toluene-stored brain respectively (Fig. III.5).

#### b. Buffer solubilized enzyme from frozen and toluene-stored brain

These two preparations also showed inhibition by excess of substrate, with the optimum at 2 mM of acetylcholine concentration but the inhibition degree was 20% for enzyme from frozen brain and just 5% for toluene-stored brain. The Km values were now 91 and 100 μM for fresh and toluene-stored brain (Fig. III. 6a).

#### c. Triton solubilized enzyme from toluene-stored and frozen brain

These two preparations were similar to the corresponding

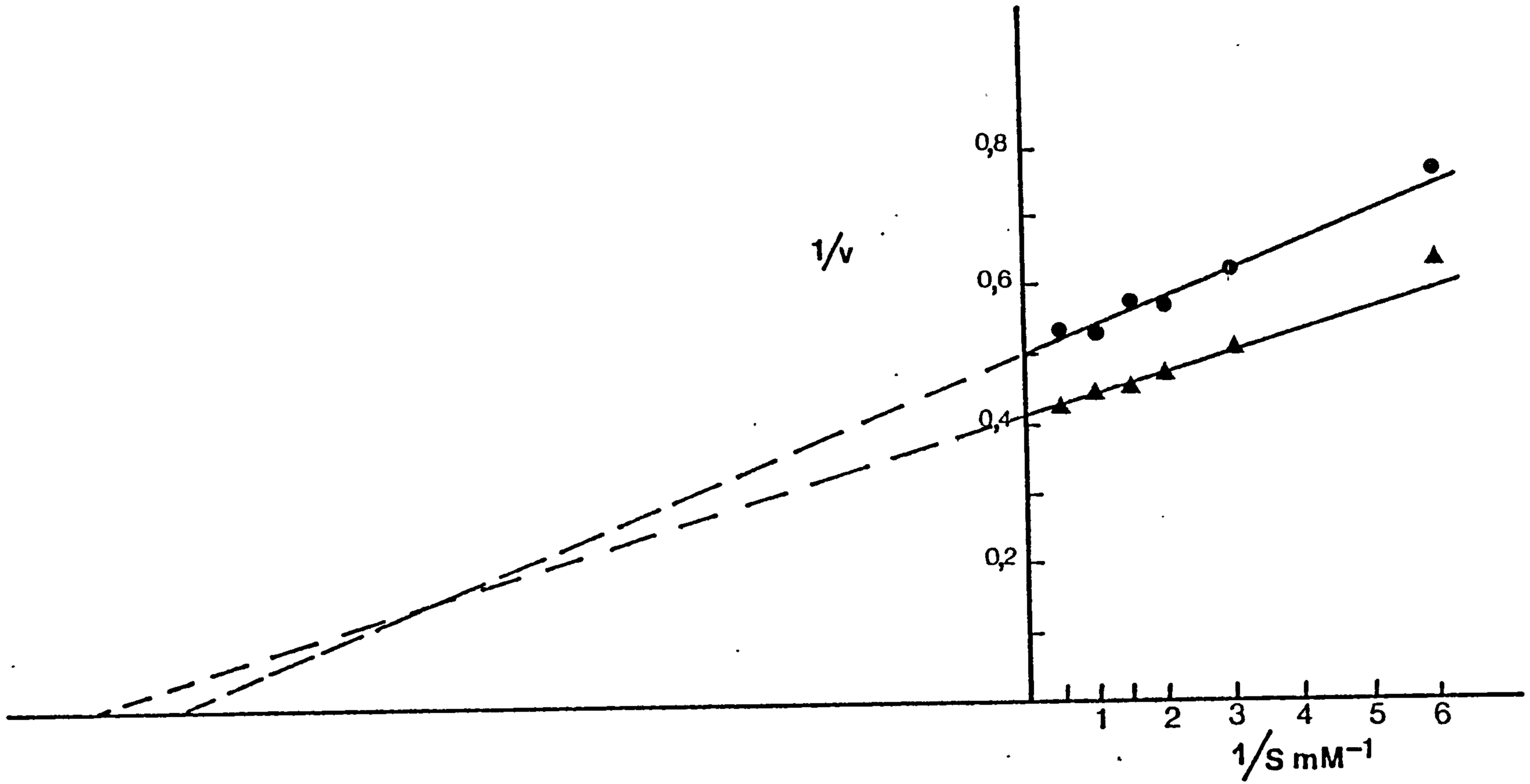



Fig. III.5
Lineweaver-Burke plots for membrane preparations from fresh (●) and toluene-stored (▲) brains.

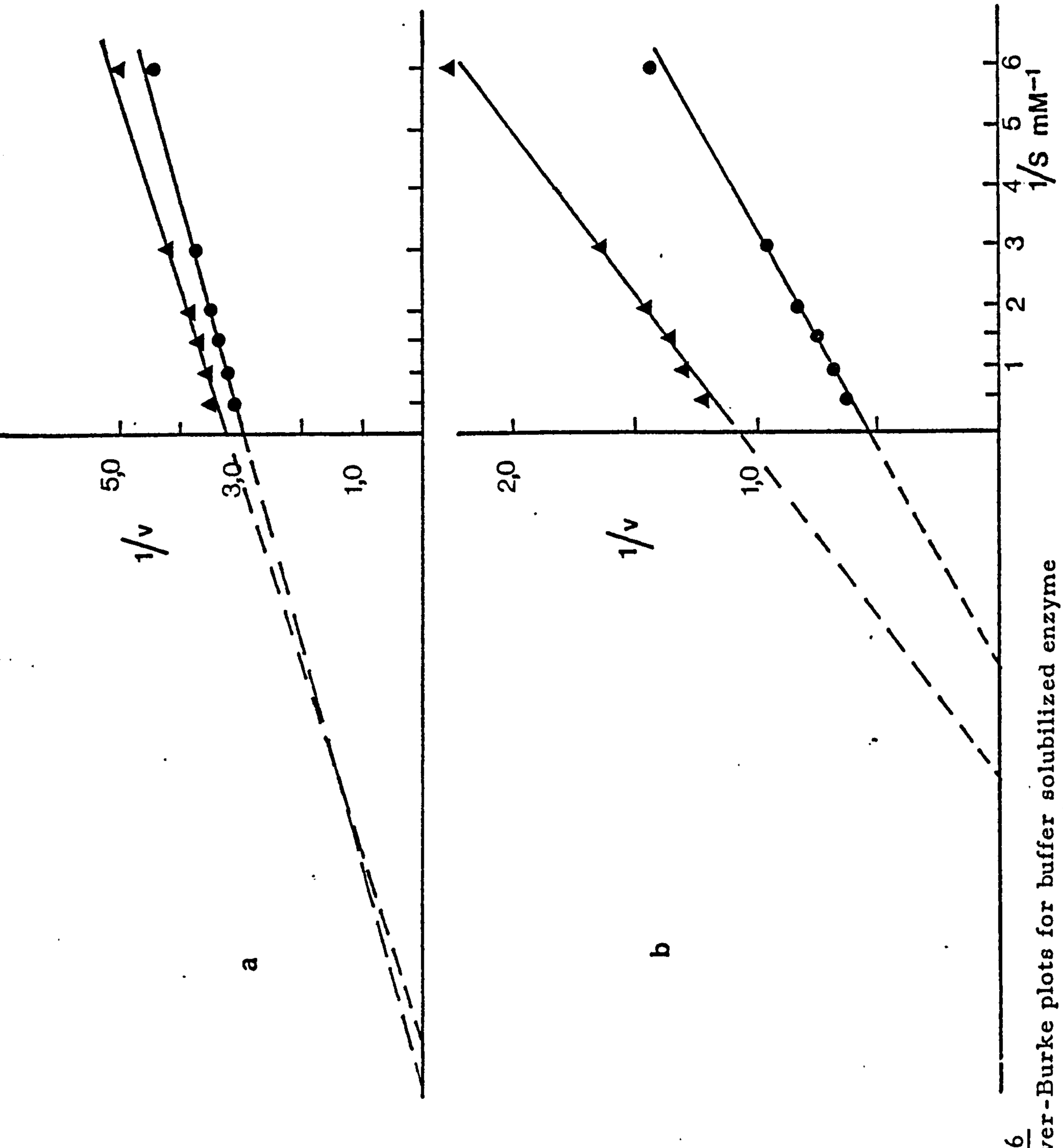



Fig. III.6
Lineweaver-Burke plots for buffer solubilized enzyme (a) and Triton-solubilized AChE (b) from fresh (●) and toluene-stored (▲) brains.

homogenates on inhibition by excess of substrate. Inhibition of about 10 and 35% for Triton solubilized preparations from frozen and toluene stored brain were measured. The Km values were 250 and 170 μM for the corresponding preparations from fresh and toluene-treated brain (Fig. III. 6b).

d. Partially purified enzyme

This preparation also presented inhibition by excess of substrate. About 25% of inhibition was measured if the substrate concentration was 20 mM. The Km value for this preparation was 206 μM.

2. Arrhenius Plots and Activation Energy

As in previous experiments, the pH-stat method was used to get the Arrhenius plots of all the different preparations. This time, the acetylcholine iodide concentration ranged from 2 mM to 0.17 mM and temperatures between 10 and 40°C. The values for Vmax and Km were found from S/v versus S plots calculated on an Elliot 4130 computer, and then log Vmax and log 1/Km were plotted against the reciprocal of the absolute temperature (Plummer, Reavill and Mc McIntosh, 1975).

a. Membrane suspensions

In these experiments membrane suspensions, either from frozen or toluene-stored rat brain, were investigated. The Arrhenius plot of membrane suspension from frozen rat brain did not show a break but in contrast the suspension prepared from

toluene-stored brain showed a pronounced break at about 25°C the transition temperature (Fig. III.7). The energies of activation were calculated as 11.4 KJ/mol for fresh brain and 28.4 KJ/mol (low temperature range) and 12.9 KJ/mol (high temperature range) for the corresponding preparations of toluene-stored brain (Table III.6.7).

b. Buffer solubilized preparations

When the enzyme was extracted with dilute buffer a break at 25°C was also observed either from frozen or toluene-stored brain. (Fig. III.8). In the first case the values for the activation energies were 11.6 KJ/mol and 4.9 KJ/mol, at low and high temperature respectively. The corresponding values for toluene-stored brain enzyme were 22.4 KJ/mol and 3.9 KJ/mol (Tables III.6 and 7).

c. Triton solubilized preparations

As before, the existence of a break at 25°C was observed from the studies on these two preparations. The values for the activation energies were 14.5 and 5.8 KJ/mol for the Triton solubilized enzyme from frozen brain and 27.4 KJ/mol and 8.7 KJ/mol for the toluene-stored preparation (Fig. III.9). The values for activation energies at low and high temperatures are summarised in the Tables III.6 and 7.

In Fig. III.10 it can be seen that there was very little change in Km values with respect to the temperature. Even bearing in mind that the standard error for Km values was as much as $\pm$20% it is quite clear that there was no break and therefore the existence of forms with different Km could be discounted. In the Tables III.8 and 9 extrapolated values for Km at low (10°C) and high temperature (40°C) for all the preparations are presented.

TABLE III. 6

Activation Energies (K Joules/mol) for preparation obtained from frozen rat brain

| Enzyme preparation | Low temperature | High temperature |
|---|---|---|
| Membrane suspension | 11.4 | 11.4 |
| Buffer solubilized | 11.6 | 4.9 |
| Triton solubilized | 14.5 | 5.8 |

TABLE III. 7

Activation Energies (K Joules/mol) for preparations obtained from toluene-stored rat brain

| Enzyme preparation | Low temperature | High temperature |
|---|---|---|
| Membrane suspension | 28.4 | 12.9 |
| Buffer solubilized | 22 4 | 3.9 |
| Triton solubilized | 27.4 | 8.7 |

TABLE III. 8

Km values ($\mu$M) at 10 and 40°C for enzyme preparations from frozen rat brain

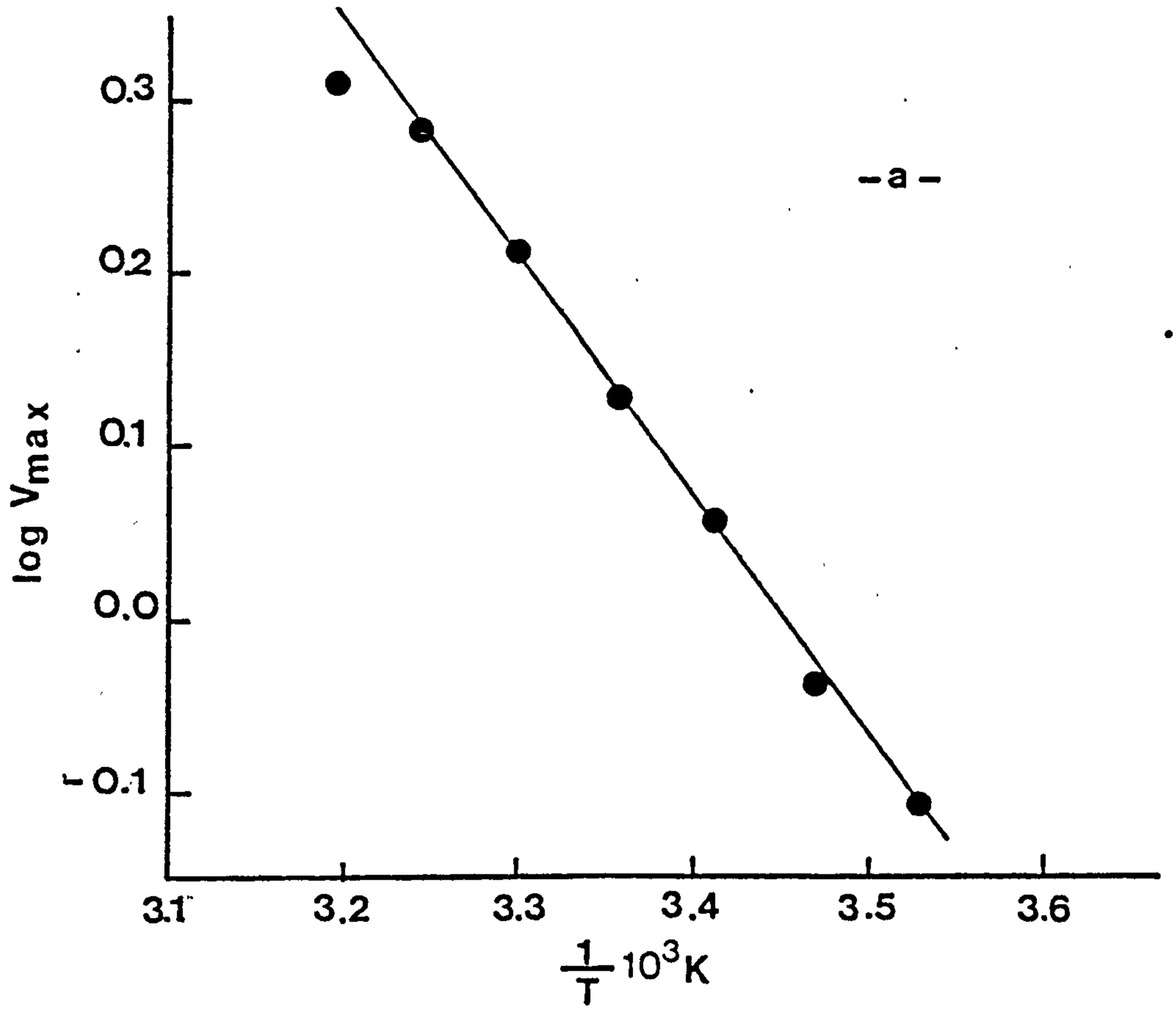
| Enzyme preparation | 40°C | 10°C |
|---|---|---|
| Membrane suspension | 85 | 46 |
| Buffer solubilized | 130 | 87 |
| Triton solubilized | 260 | 131 |

TABLE III. 9

Km values ($\mu$M) at 10 and 40°C for enzyme preparation from toluene-stored rat brain

| Enzyme preparation | 40°C | 10°C |
|---|---|---|
| Membrane suspension | 150 | 100 |
| Buffer solubilized | 100 | 80 |
| Triton solubilized | 175 | 130 |

Fig. III.7
Arrhenius Plots with membrane preparations.
a. - Frozen brain; b. - Toluene-stored brain.



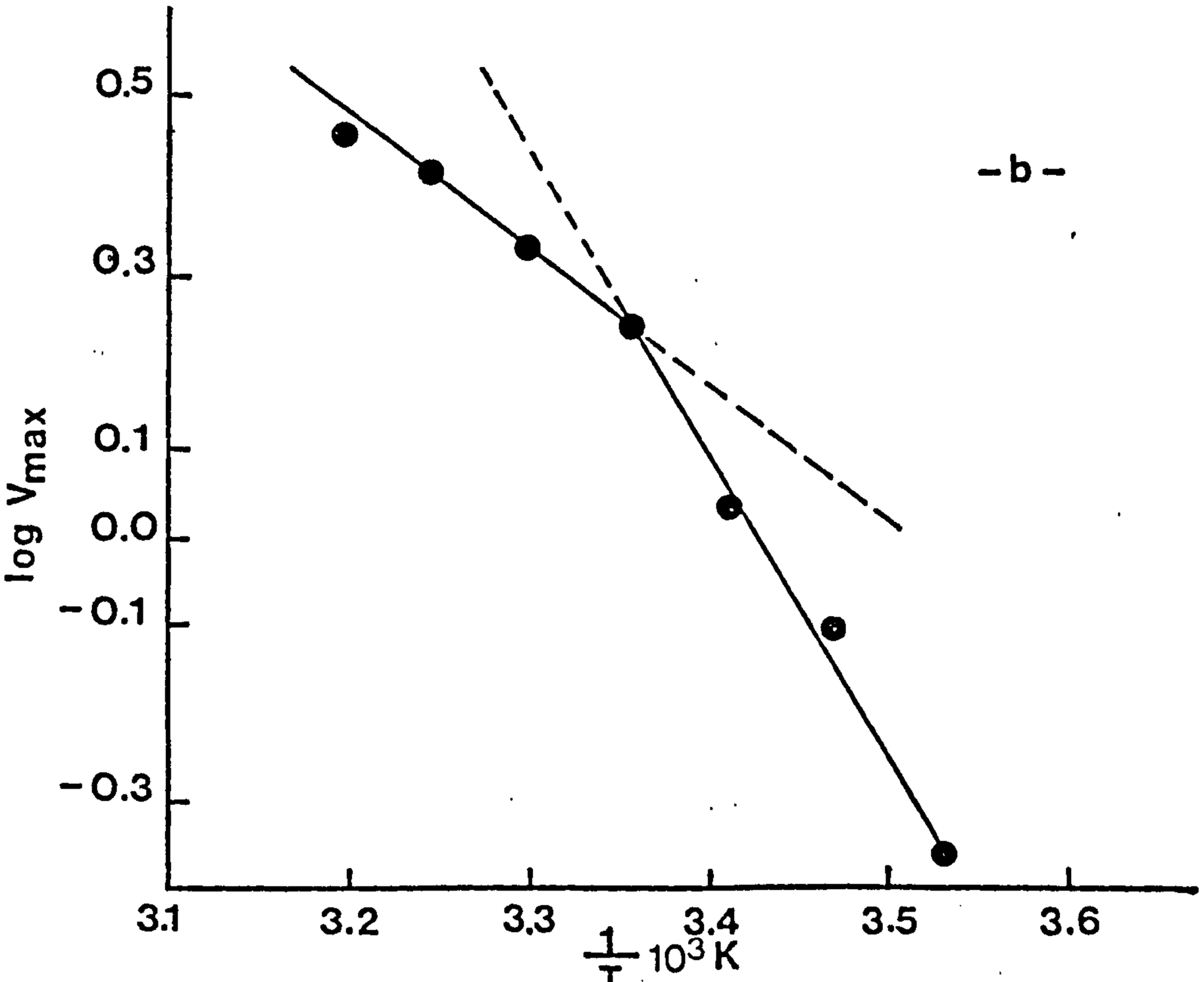



Fig. III.8
Arrhenius Plots of buffer-solubilized preparations.
a. Frozen brain; b. Toluene-stored brain.

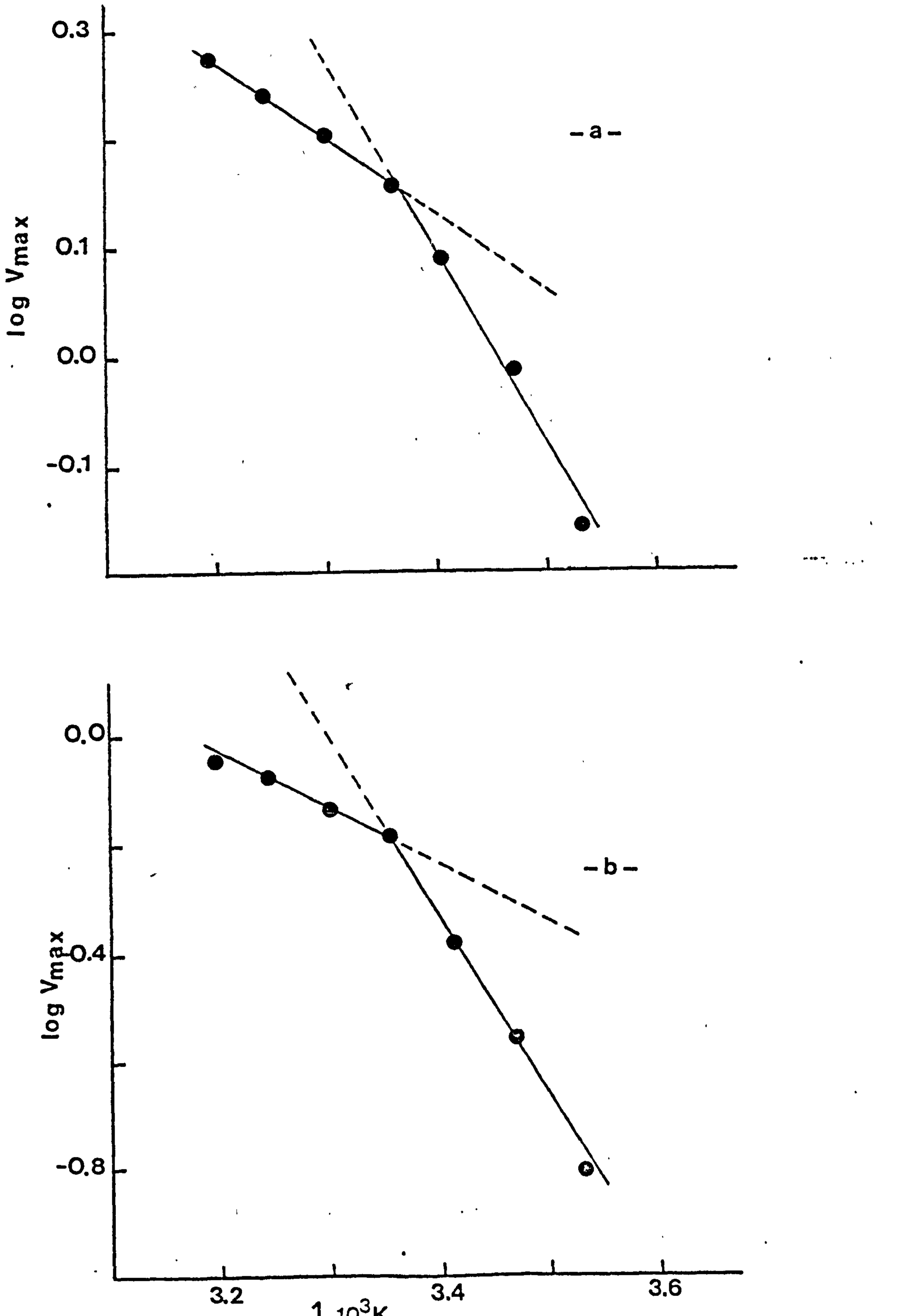
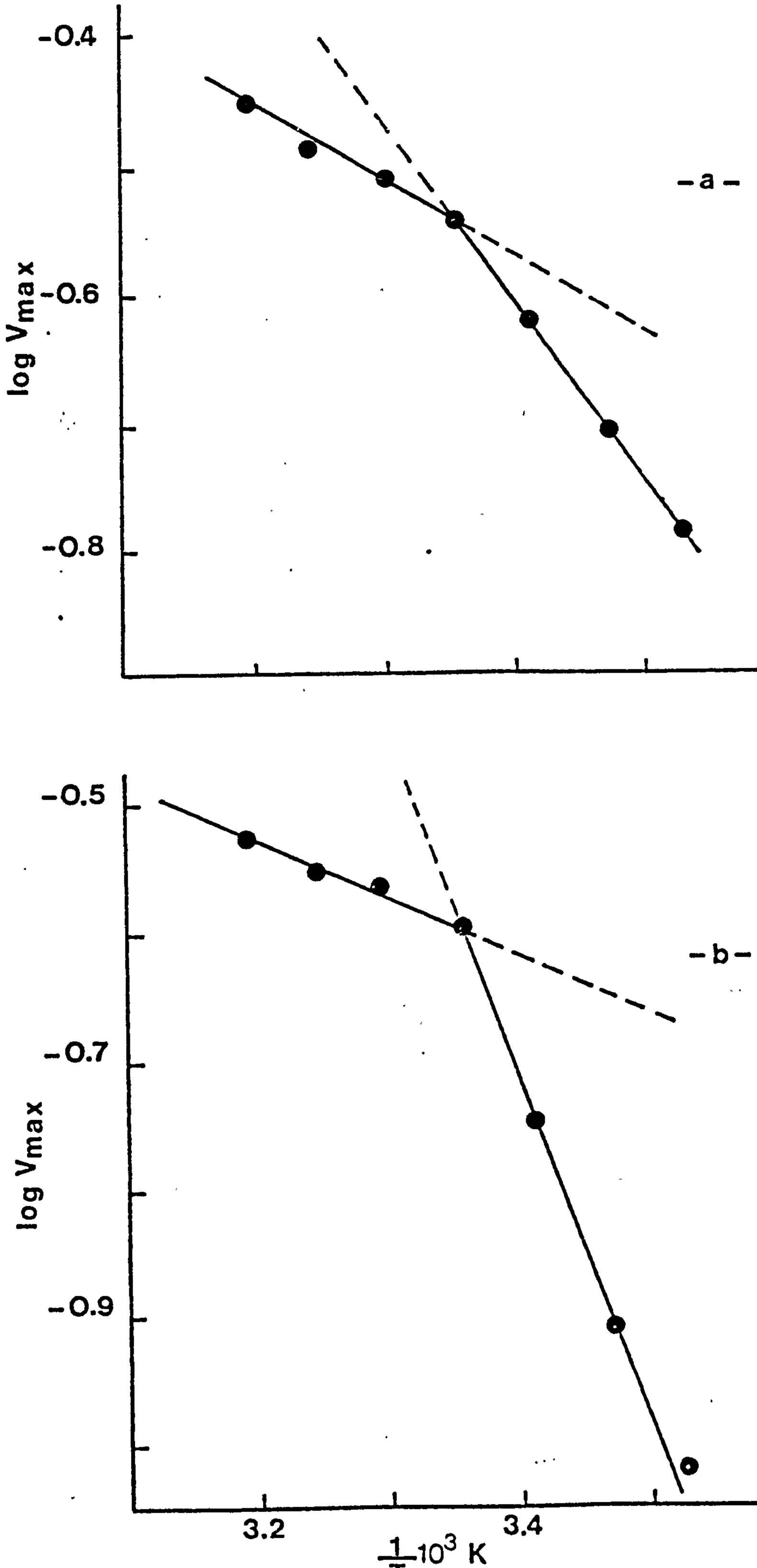


Fig. III.9
Arrhenius plots of Triton-solubilized preparations.
a. - Frozen brain; b. - Toluene-stored brain.



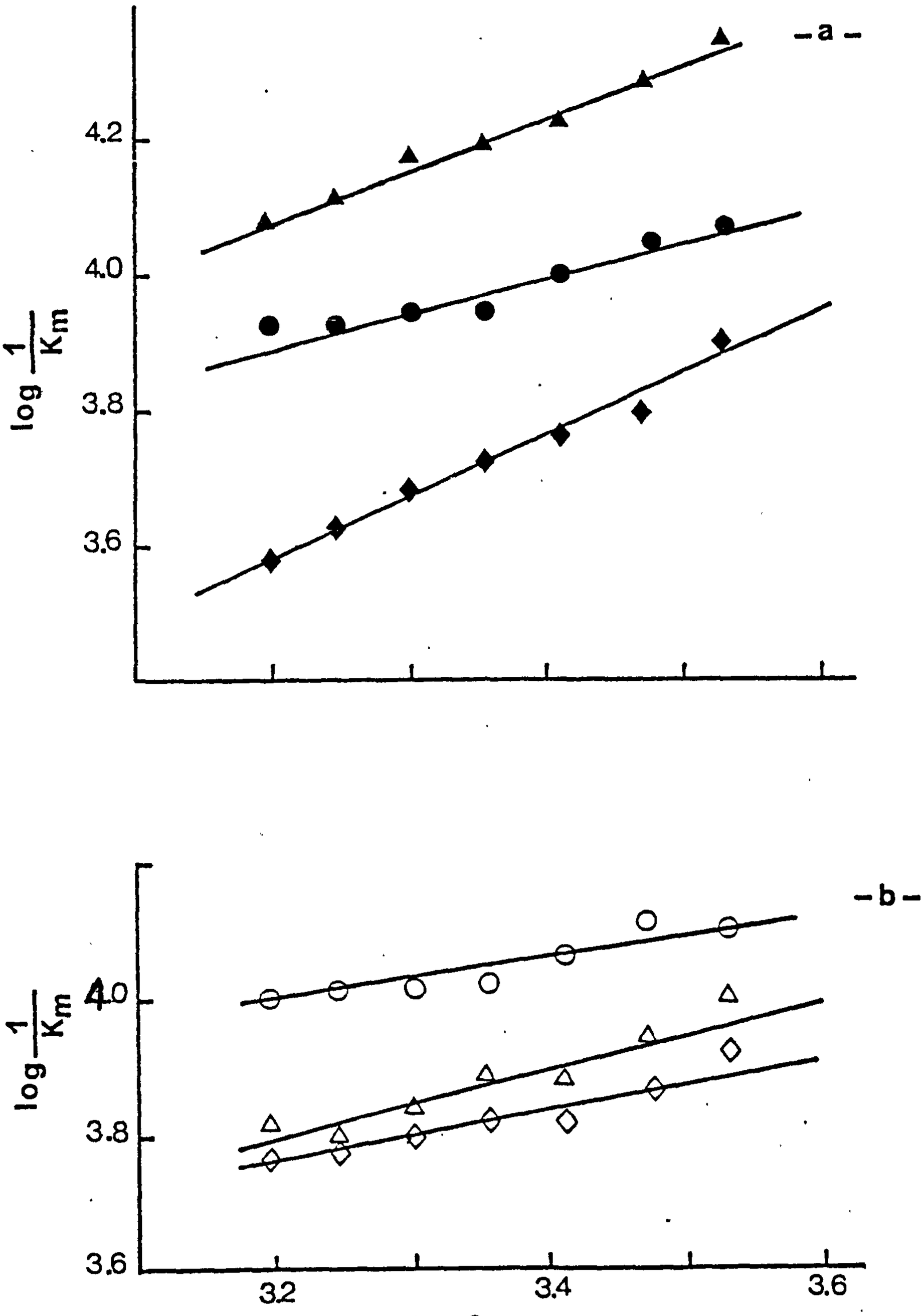



Fig. III.10

Km dependence with temperature; a. Frozen brain; b. Toluene-stored brain; ▲ △ Homogenate; ● ○ Buffer solubilized; ◆ ◇ Triton solubilized.

## E. SEPARATION OF MULTIPLE MOLECULAR FORMS BY DIFFERENCES IN ELECTRICAL CHARGE

### 1. Starch Block Electrophoresis

#### a. Acetylcholinesterase from frozen rat brain

When the Triton solubilized enzyme was electrophoresed on a 20 cm starch block for 18 h and phosphate buffer pH 8, 0.1M (Triton 1%) as work buffer, only one peak of activity was resolved which was very slow moving (Fig. III.11a). In order to get better resolution the running time was increased to 50 h and at the end of that period a gradient of pH along the block was found. Indeed, the enzyme was denatured and in the eluted fractions precipitation or turbidity was observed in the portion of block corresponding to a range of pH 10-2.2 (Fig. III.11c). To avoid this problem a longer perspex former was used (30 cm) and buffers with different ionic strengths were tested. When the Triton-solubilized enzyme was run on a 30 cm starch block for 66 h and 0.2M phosphate buffer pH 8, (Triton 1% w/v), the resolution was extremely good and 5-6 bands were detected. It is important to note that all of them were very slow-moving and therefore very close. All the forms were eluted from the portion of the block located in between the origin and the haemoglobin. The recovery was nearly 50% (FigIII12a). When the same preparation was electrophoresed for 48 h using phosphate buffer pH 8 0.1M, Triton X-100 1%, a very similar pattern to the previous one was obtained. As in that case 5-6 bands were detected in between the origin and haemoglobin, but now the recovery was lower, only 25% (Fig. 13a). Finally the preparation was run for

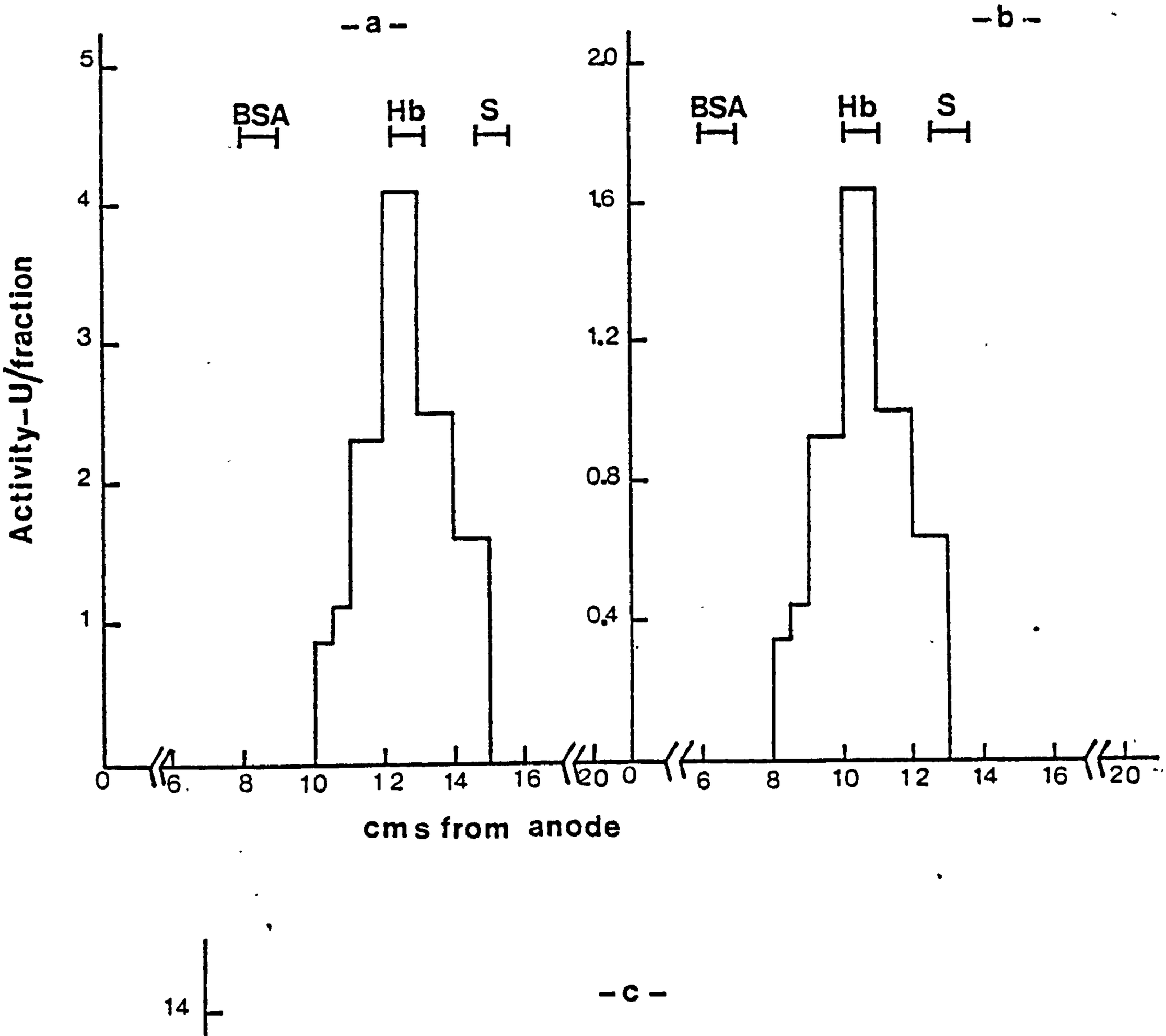
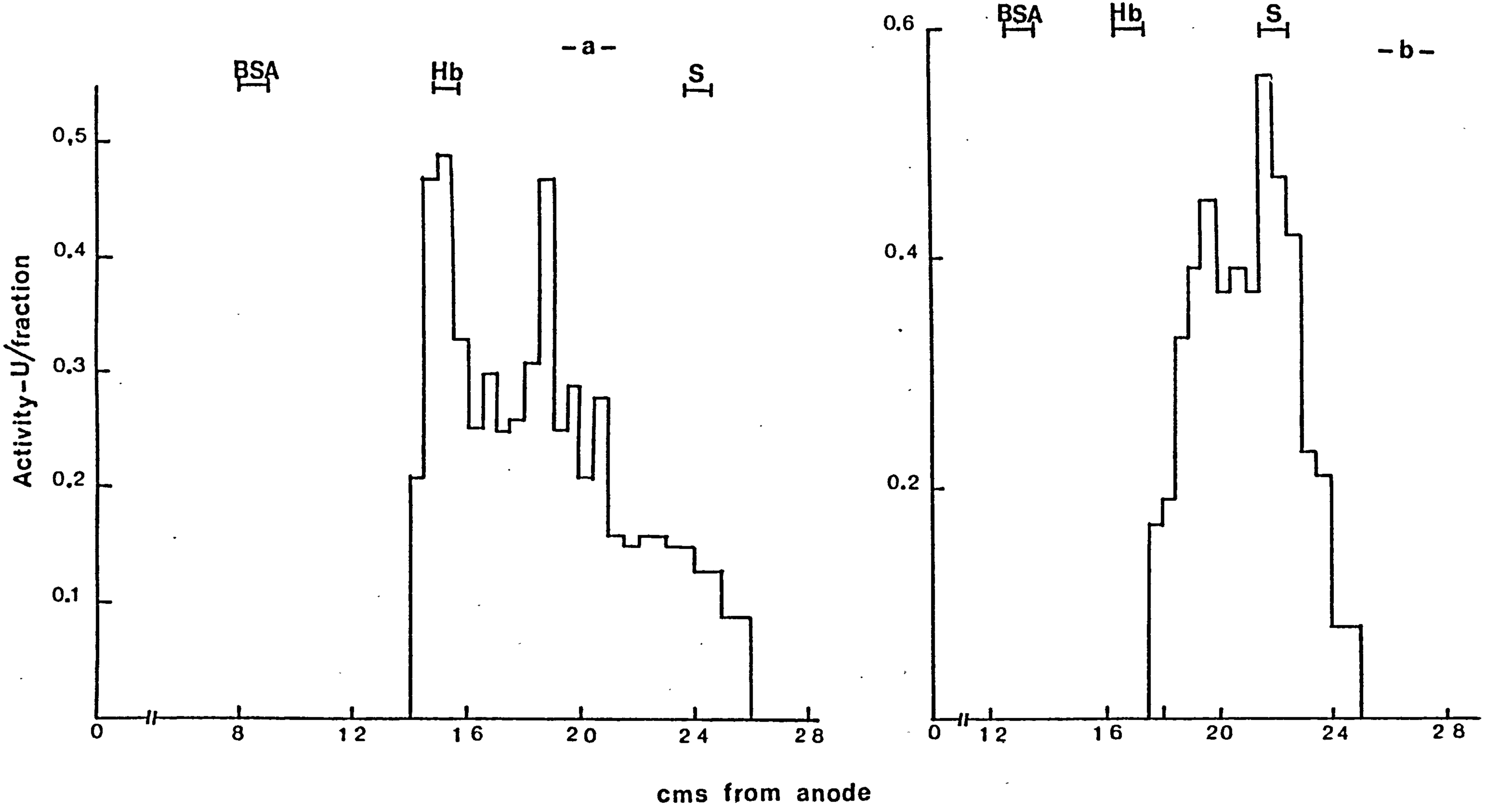



Fig. III.11
Starch block electrophoresis: a. Triton solubilized enzyme from frozen brain. V = 110 vol, I = 80 mA, Time 20 h; b. Triton solubilized enzyme from toluene-stored brain. V = 90 vol, I = 90 mA, Time 24 h; c. As a. Time 50 h.

Fig. III.12

Starch block electrophoresis. Triton solubilized enzyme from frozen brain. a. sodium phosphate buffer 0.2M pH 8.0; V = 90 vol; I = 120 mA; Time 66 h. b. Sodium phosphate - 0.05M pH 8.0; V = 210 vol; I = 100 mA; Time 24 h.



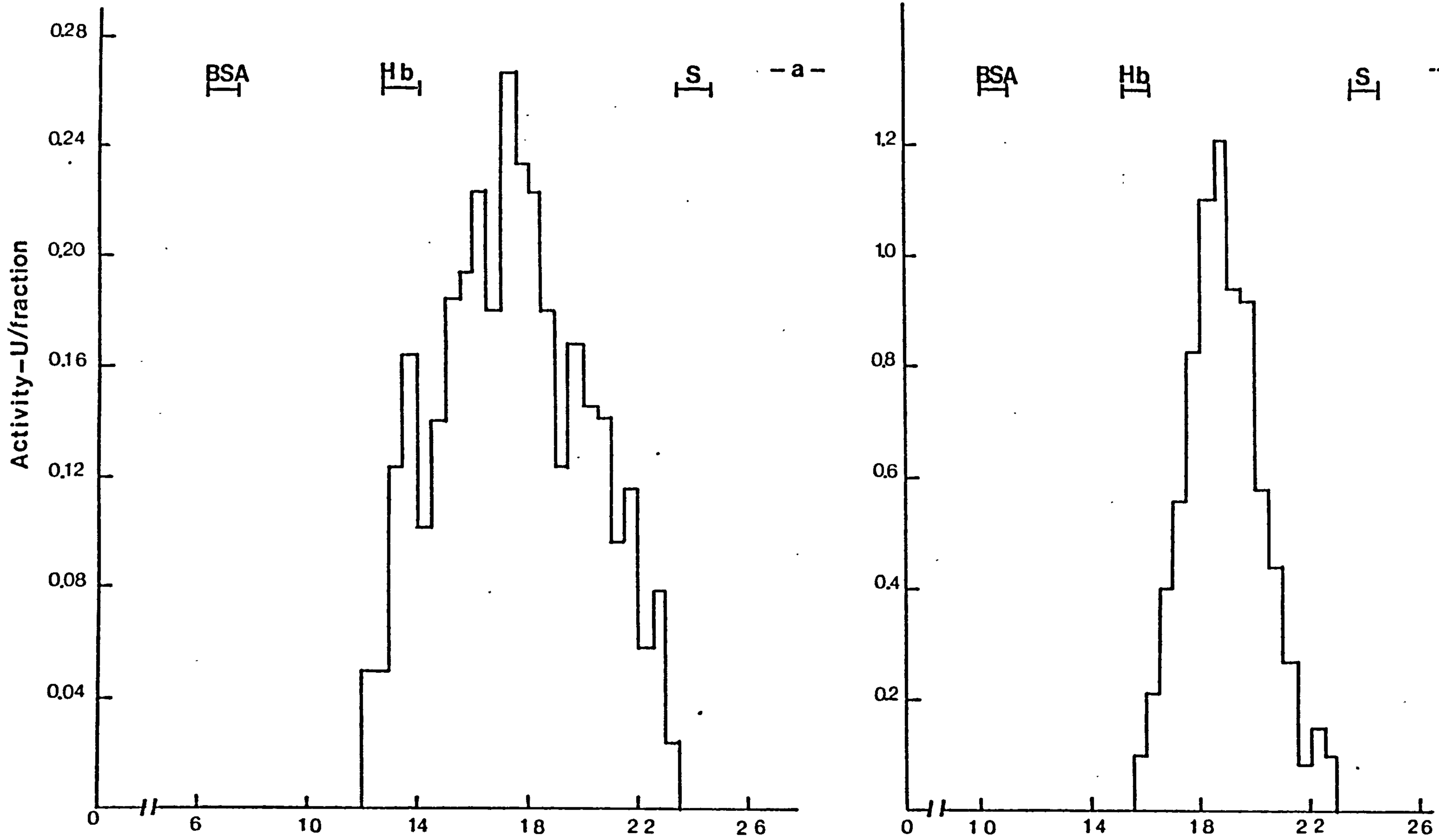



Fig. III.13
Starch block electrophoresis: a. Triton solubilized enzyme from frozen brain. P-P buffer 0.1M pH 8.0; I = 120 mA, V = 160 vol, Time 48 h. b. Triton solubilized enzyme from toluene-stored brain. Buffer as a. I = 120 mA, V = 150 vol Time 36 h.

24 h, using phosphate buffer pH 8, 0.1M, Triton X-100 1% and under these conditions, lower resolution than with the previous ones was achieved (Fig. 12b). The bands were detected in the same position as before and the recovery was around 30%.

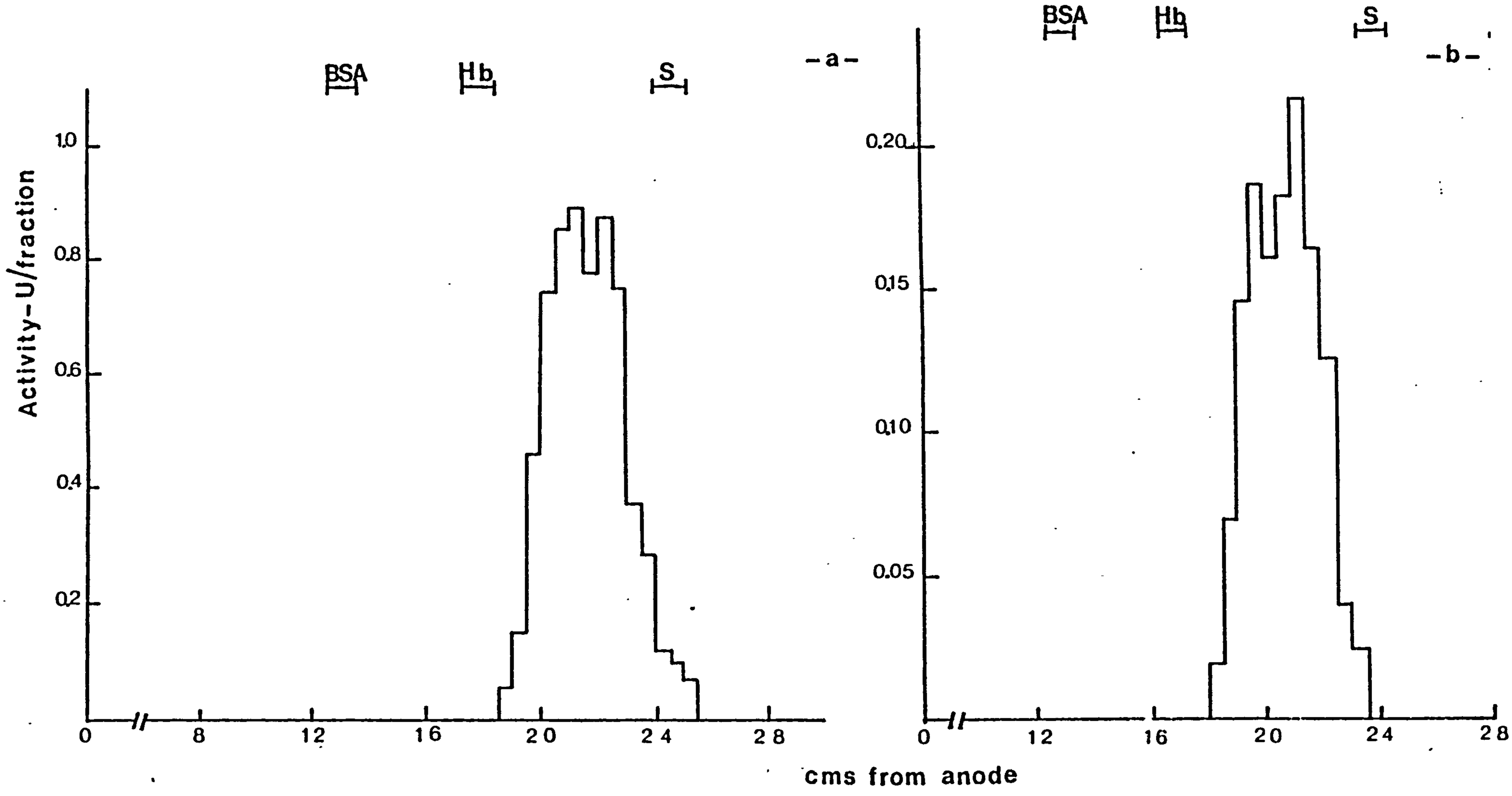
It was therefore decided that the optimal conditions to run the enzyme were phosphate buffer 0.1M pH8 with or without Triton X-100 depending on the sample, and for long enough to obtain a good separation in between the markers. When buffer-solubilized enzyme, 'naturally soluble', was electrophoresed for 24 h, and phosphate buffer pH 8, 0.1M only 2-3 bands were detected in between the origin and the position corresponding to the haemoglobin. The recovery was nearly 85% (Fig. III.14a).

b. Acetylcholinesterase from toluene-stored brain

When Triton-solubilized enzyme was electrophoresed for 18h on a 20 cm starch block using phosphate buffer pH 8 0.1M 1% Triton an identical result to that obtained with enzyme from frozen rat brain was produced. If the same sample was applied on a 30 cm starch block for 36 h and higher voltage, better resolution could be achieved but not as good as that in the case of frozen rat brain (Fig. 13b). The recovery was 90%. When the buffer solubilized enzyme was used under the same conditions, better resolution was observed but this time the recovery dropped to 35% (Fig. III.14b).

The homologous fraction were separated for Triton-solubilized enzyme from frozen rat brain and the most active was re-electrophoresed under the same standard conditions, showing a unique fraction, with its corresponding position in between markers.

Fig. III.14
Starch block electrophoresis: a. Buffer solubilized enzyme from frozen brain. I = 110 mA; V = 150 vol; Time 24 h; b. Buffer solubilized enzyme from toluene-stored brain. I = 180 mA; V = 100 vol; Time 24 h.



## 2. Polyacrylamide Gel Electrophoresis in Rods

### a. Frozen tissue

The enzyme band patterns are shown in Fig. III.15. The Triton solubilized enzyme gave six main bands and two quite faint ones when the electrophoresis was carried out at 4°C but at 40°C only four bands were detected. On the other hand, the naturally soluble enzyme presented 4 bands at 4°C and about the same pattern at 40°C (Fig. III.15).

### b. Toluene-stored tissue

The Triton solubilized enzyme gave 4 bands of activity, one of them quite faint at 4°C and an almost identical pattern was observed at 40°C. When buffer solubilized enzyme was used, three main bands and two faint ones were detected but at 40°C that number was drastically reduced to one main band and one faint one (Fig. III.15).

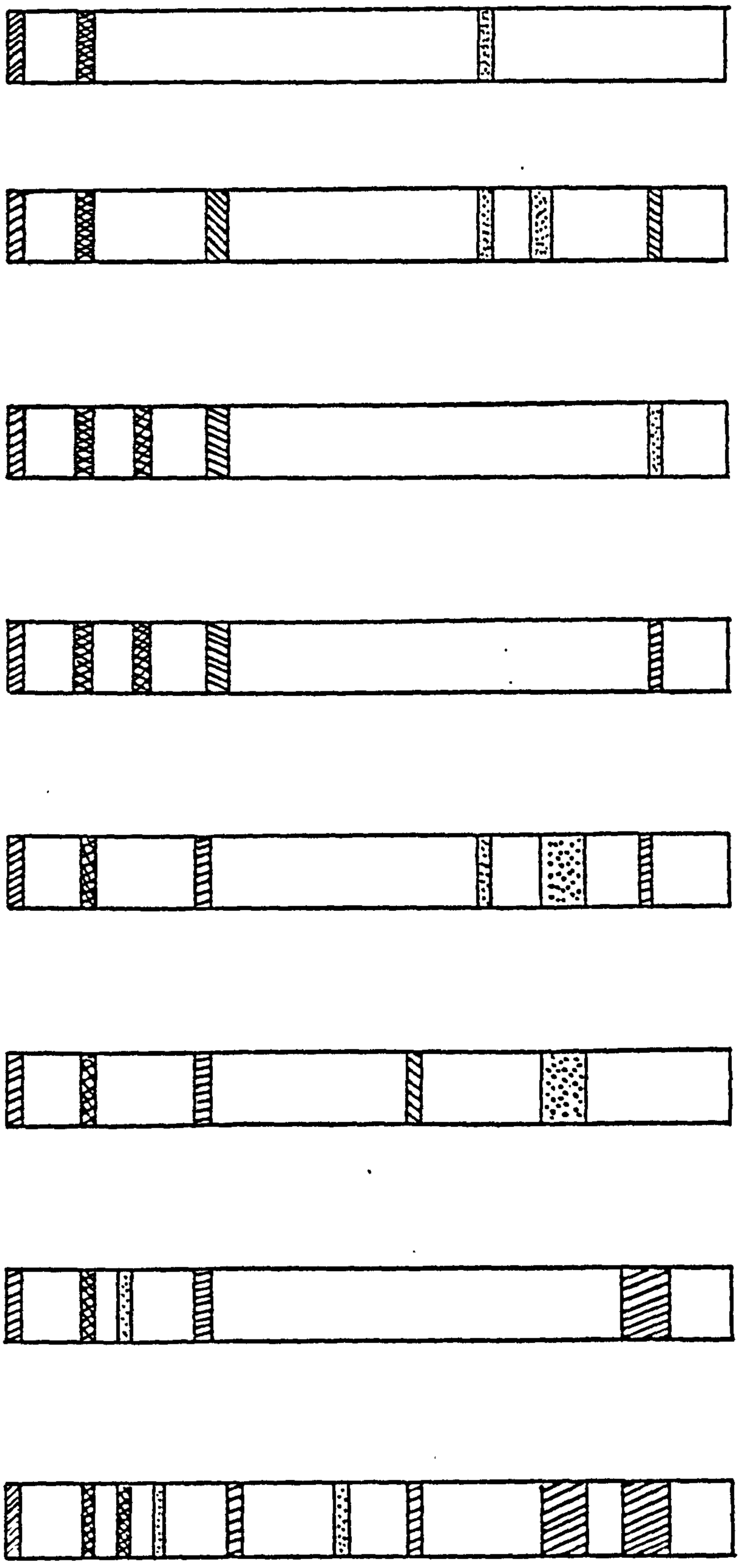



Fig. III.15
Electrophoresis in rods of polyacryamide gel (7%): Triton solubilized enzyme from frozen (1) or toluene-stored brain (2). Buffer solubilized enzyme from frozen (3) or toluene-stored brain (4). a. Electrophoresis carried out in cold room (+4°C) b. Electrophoresis carried out in constant temperature room (40°C). Depth of staining was: ▨ strong; ▨ moderate; ▨ light.

## F. SEPARATION OF MULTIPLE FORMS BY DIFFERENCES IN SIZE

### 1. Sucrose Density Gradient Centrifugation

#### a. Standard enzyme preparations

When three standard proteins were run on density gradients there was found to be a direct relationship between the sedimentation coefficients and distance migrated down the tube for sucrose gradients. (Fig. III.16). This relationship was used in future centrifugations by comparing the migration distances of catalase and AChE.

In this set of experiments, all samples were concentrated by Minicon Concentrators about 20 times and an inactivation between 25-30% was detected in all cases.

(i) Toluene treated brain (Fig. III.17). When preparations of buffer solubilized or buffer containing Triton X-100 from toluene-stored brain were centrifuged on sucrose gradient three forms were found with sedimentation coefficients of 14.5-15.2S; 10.1-10.3S; 8.8-7.8S in both cases, corresponding to molecular weights of 350,000-370,000, 200,000 - and 165,000-135,000. However, the relative contribution of each form was slightly different in these two preparations. From buffer dilute extracted enzyme the ratio from heaviest to lightest form was 70:5:25 and from Triton X-100 solubilized enzyme the ratio was 65:12:23. A shoulder was found of 6.3-5.5S corresponding to a weight of 98,000-80,500.

(ii) Frozen rat brain. (Fig. III.18). As before concentrated

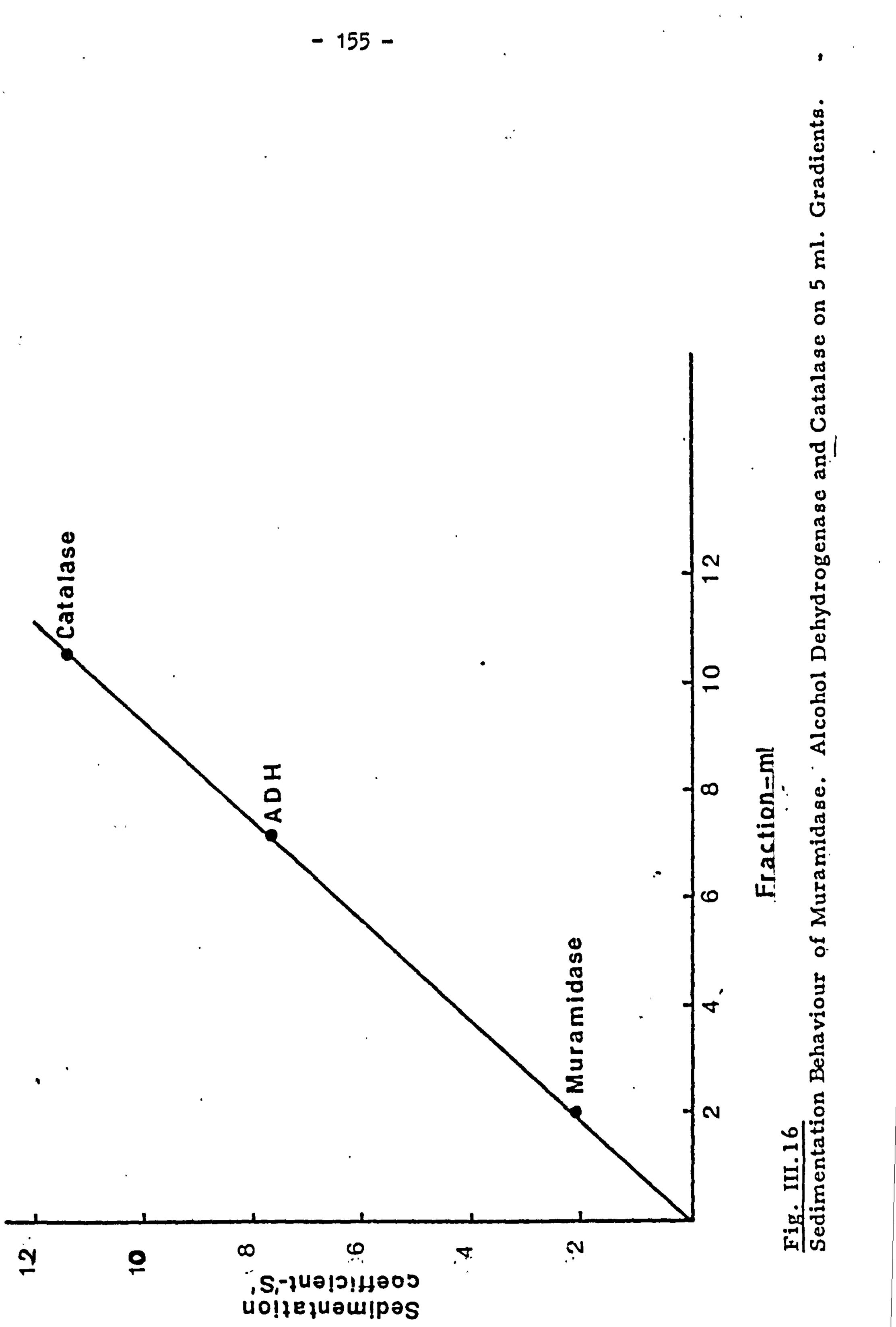
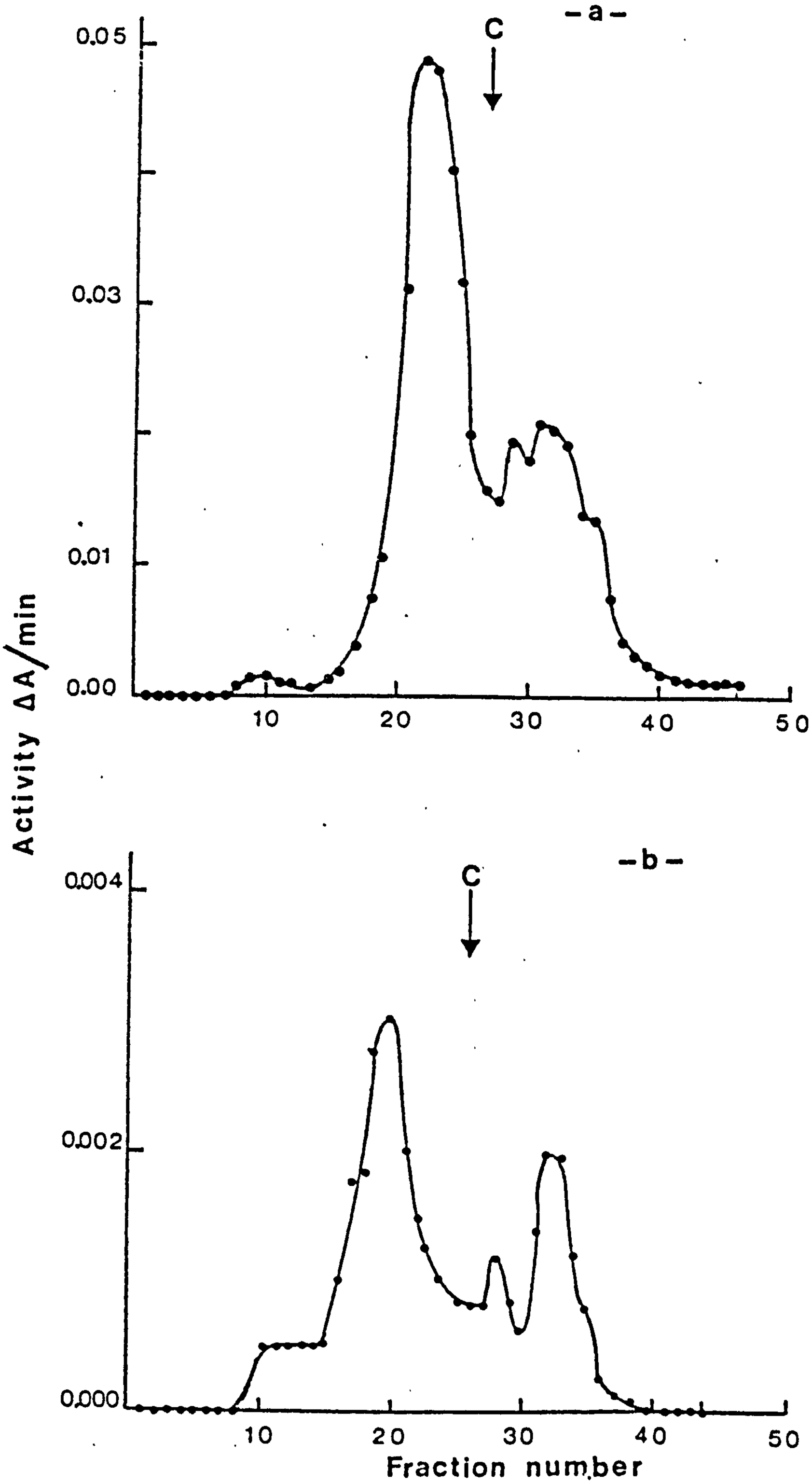



Fig. III.16
Sedimentation Behaviour of Muramidase. Alcohol Dehydrogenase and Catalase on 5 ml. Gradients.

Fig. III.17

Sucrose gradient centrifugation: Concentrated samples of toluene-stored brain. a. Triton solubilized b. buffer solubilized. The arrow shows the position of the catalase marker.



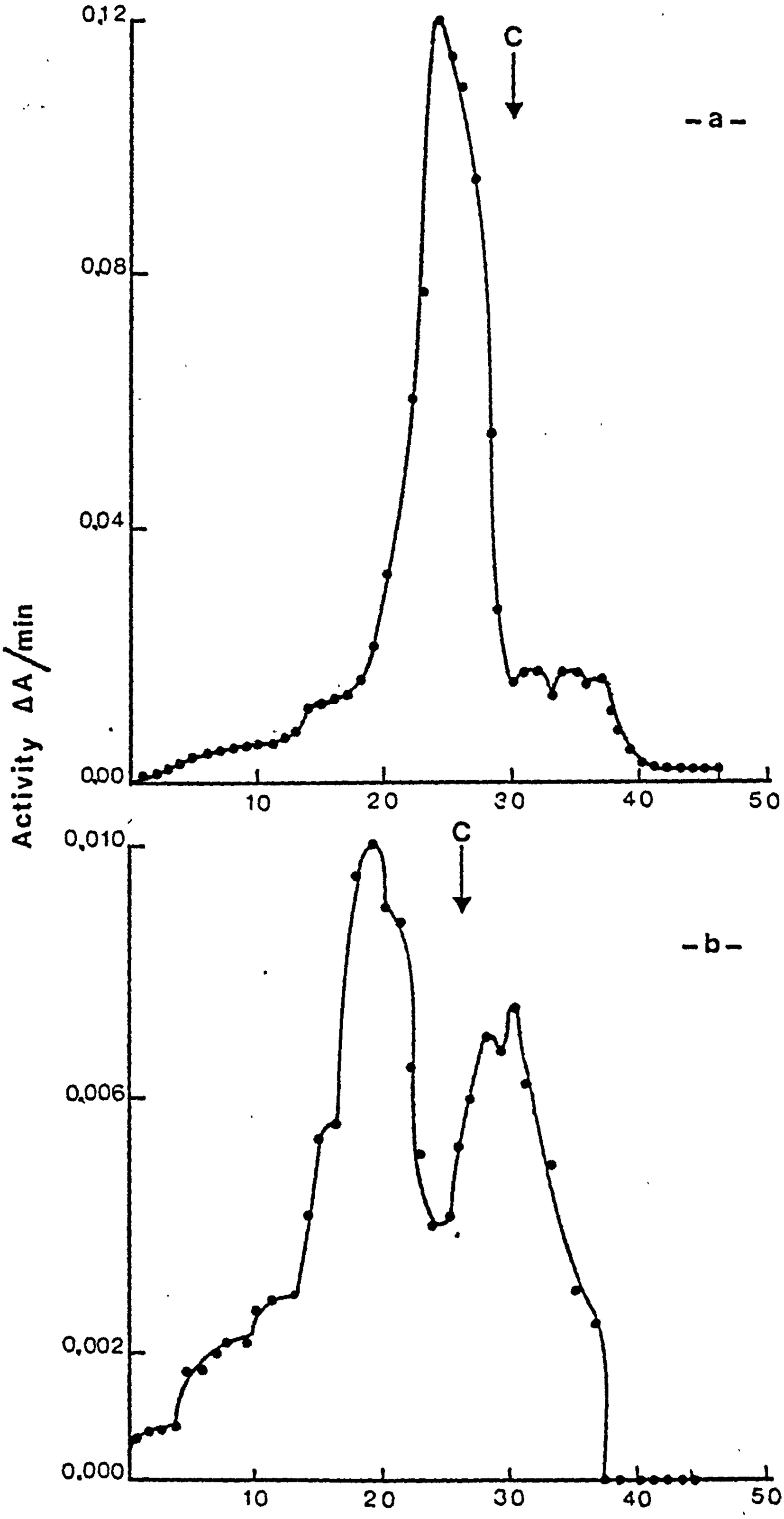
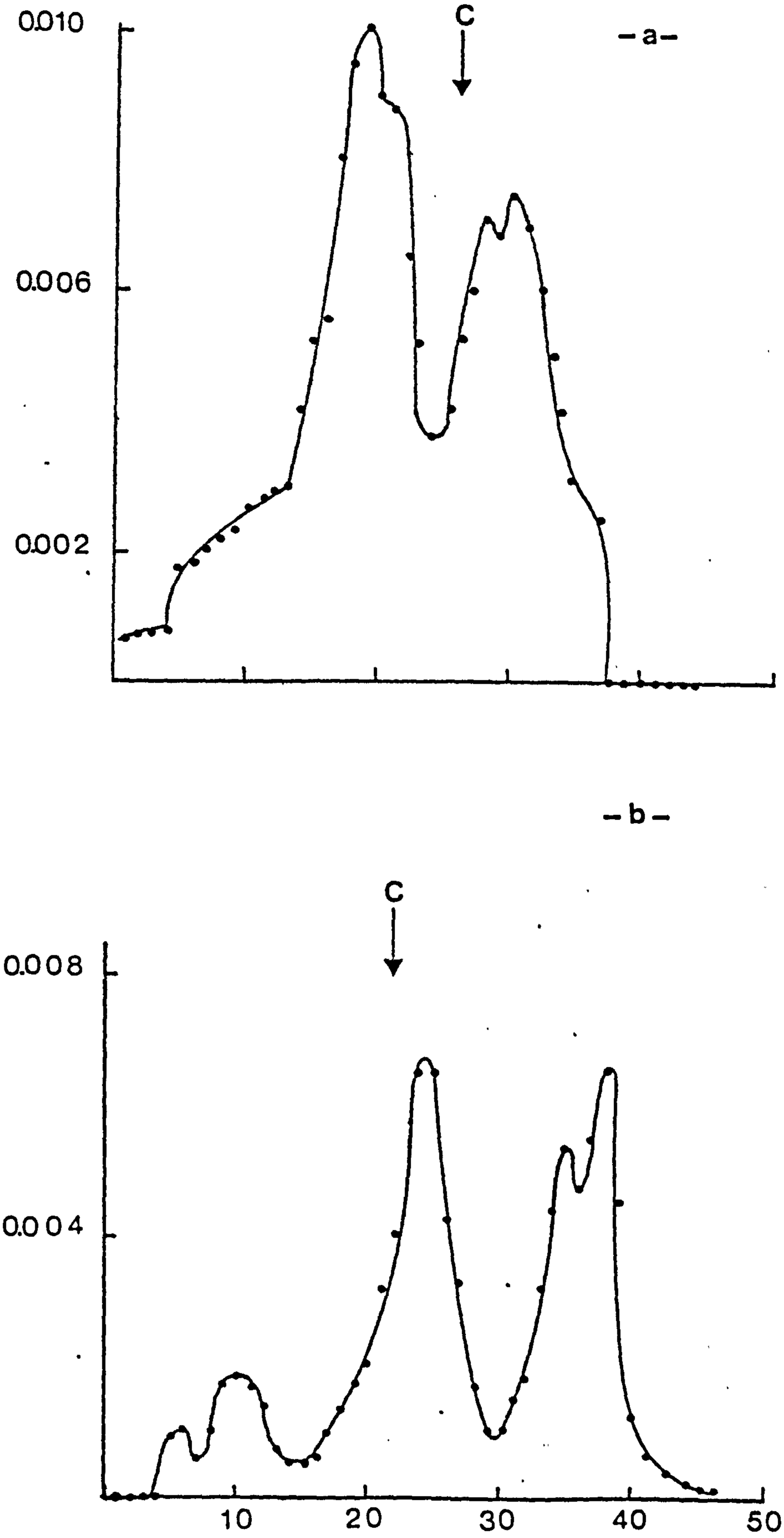



Fig. III.18

Sucrose gradient centrifugation: Concentrated samples of Triton solubilized enzyme (a) and buffer solubilized (b) from frozen brain. The arrow shows position of the marker (catalase)

Fig. III.19
Sucrose gradient centrifugation: Concentrated samples of "naturally soluble" enzyme. Before (a) and after incubation at 37°C for 24 h (b).



enzyme solubilized by dilute buffer or Triton X-100 was used and now forms with sedimentation coefficients of ~15.7S; 10S and 8.8-8.5S and molecular weights of 380,000, 200,000 and 150,000 were found. As previously a shoulder of 6.5S was detected. The relative proportion of these multiple forms was 52:25:22 for "naturally soluble" enzyme and 80:5:15 for Triton solubilized acetylcholinesterase.

b. Effect of temperature on concentrated samples

Concentrated samples of "naturally soluble" enzyme and "Triton solubilized" acetylcholinesterase from frozen rat brain were incubated at 37°C for 24h or for just half an hour, before applying to the gradients.

(i) Concentrated "naturally soluble" enzyme ( Fig. III.19). Forms with sedimentations coefficients of ~16.5S, 10.4S, 5.2S and 4S were observed with molecular weights in the range of 420,000, 210,000, 74,000 and 50,000. The ratio of these forms was 13:43:24:19. After the incubation period an inactivation of 15-20% was found.

(ii) Concentrated "Triton solubilized enzyme". (Fig. III.20-21). If the incubation time was 24 h,forms with sedimentation coefficients of ~16.0S, 10S, 6.6 and 5.2S were detected. The calculated molecular weights were 400,000, 200,000, 100,000 and 75,000. The ratio of these forms was 5:80:5:9. A loss of 10-15% of the enzyme activity was seen on incubation. When the incubation time was only half an hour inactivation of 10% was also measured. The obtained pattern was very similar to the previous one. Only instead of 5S

Fig. III.20
Sucrose gradient centrifugation. Concentrated samples of Triton solubilized enzyme from frozen brain before (a) and after incubation at 37°C for 24 h.

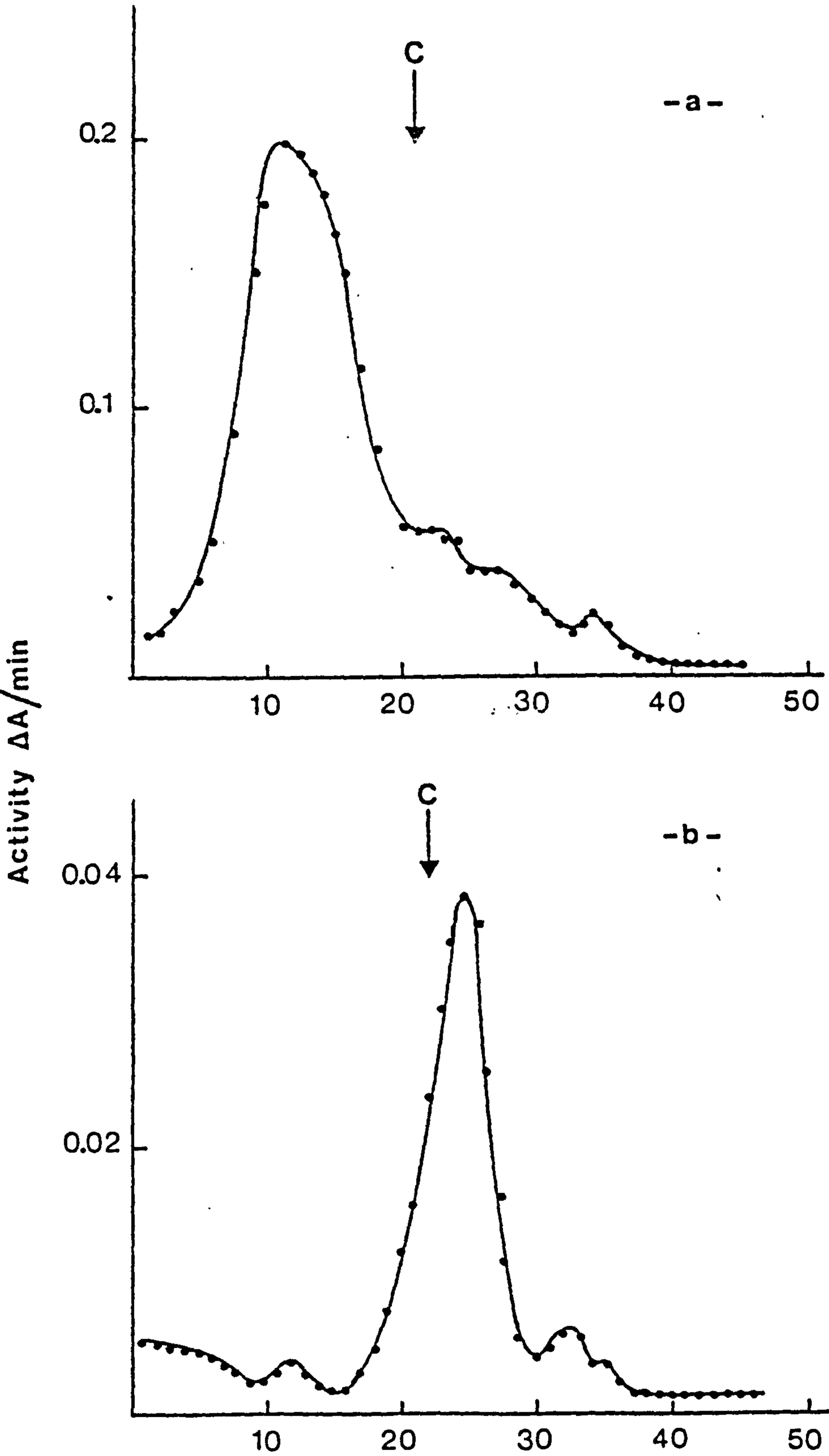
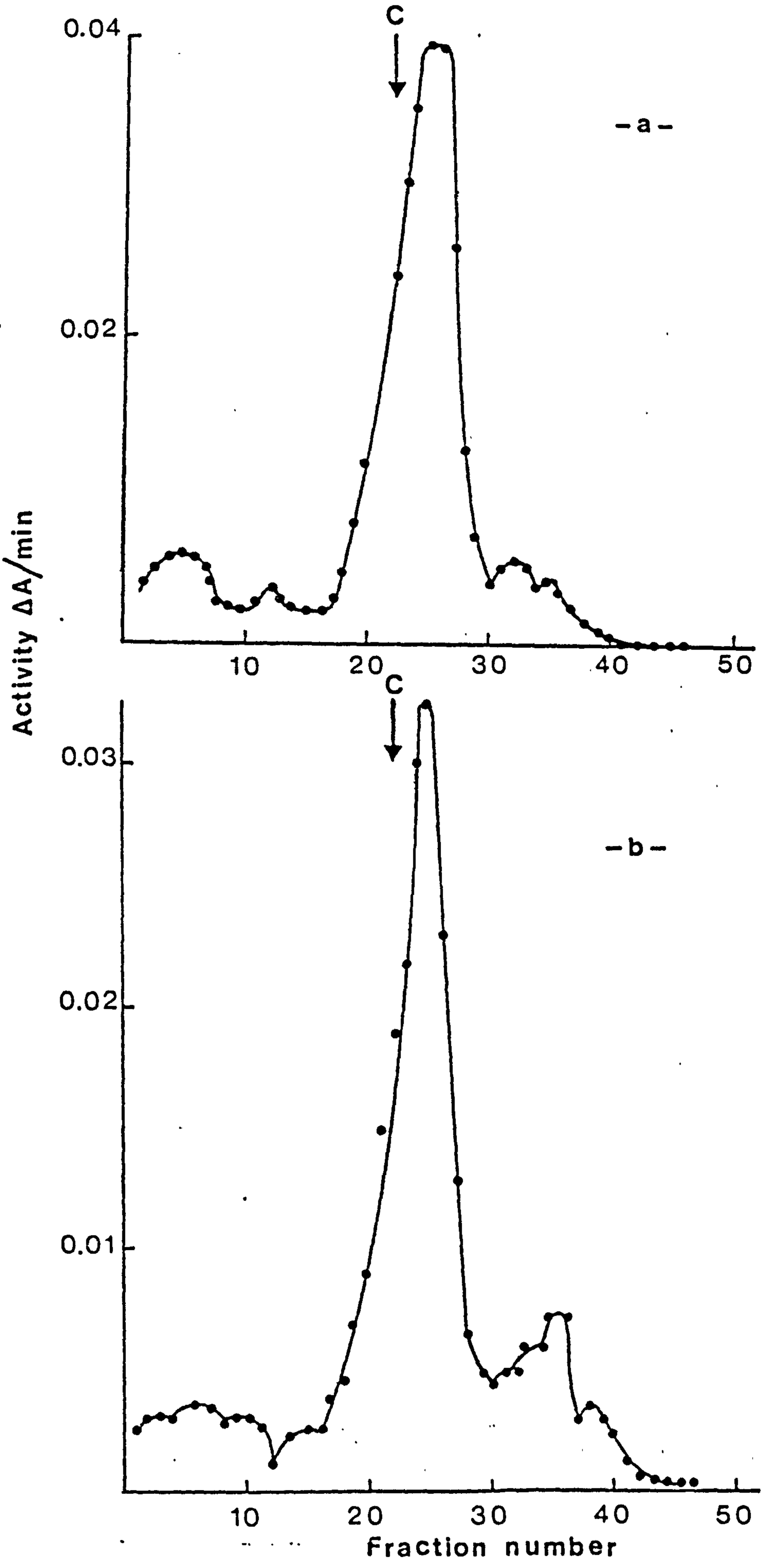


Fig. III.21
Sucrose gradient centrifugation: Triton solubilized preparation after incubation at 37°C for 24 h (a) or 30 min. (b).



another peak of 4S (50,000) was detected. The ratio of 16S, 10S, 6.6S and 6.6S and 4S forms was 10:70:14:4.

c. Effect of NaCl on concentrated samples (Fig. III.24a).

When NaCl was added to a concentrated sample of Triton solubilized enzyme, at a final concentration of 0.2M, inactivation of 15-20% was obtained. The elution profile was clearly similar to the obtained after incubation at 37°C. Again,forms of 16.1S, 10S 6S and 4.2 appear and the relatives areas were in the ratio 15:65: 11:9.

d. Effect of antiproteases agents on concentrated samples

Frozen rat brain was homogenized in buffer containing Triton X-100 (1%) in the presence of antiproteases factors (see before). The sample was concentrated and applied on gradients.

Three main forms with sedimentation coefficients of 16.4S, 9.6-10S, 7.8S and sometime 4.6 were detected (Fig. III.22-23). with approximate molecular weights of 410,000, 200,000, 150,000 and 70,000 in the ratio 45:46:4:1. However the 10S form probably was highly contaminated with the 7.8S as is shown in Fig. III.23.

If that preparation was incubated at 37°C for 24 h the pattern showed forms of 15.6S, 9.3S, 7.9S and 5.4S in the ratio 20:35:30: 15. Inactivation up to nearly 75% was found after the incubation period.

e. Influence of concentration on sucrose gradient pattern

In this set of experiments samples of "Triton solubilized"

Fig. III.22
Sucrose gradient centrifugation: Concentrated samples of Triton-solubilized enzyme in the absence of antiproteases agents (a) in the presence of antiproteases agents (b).

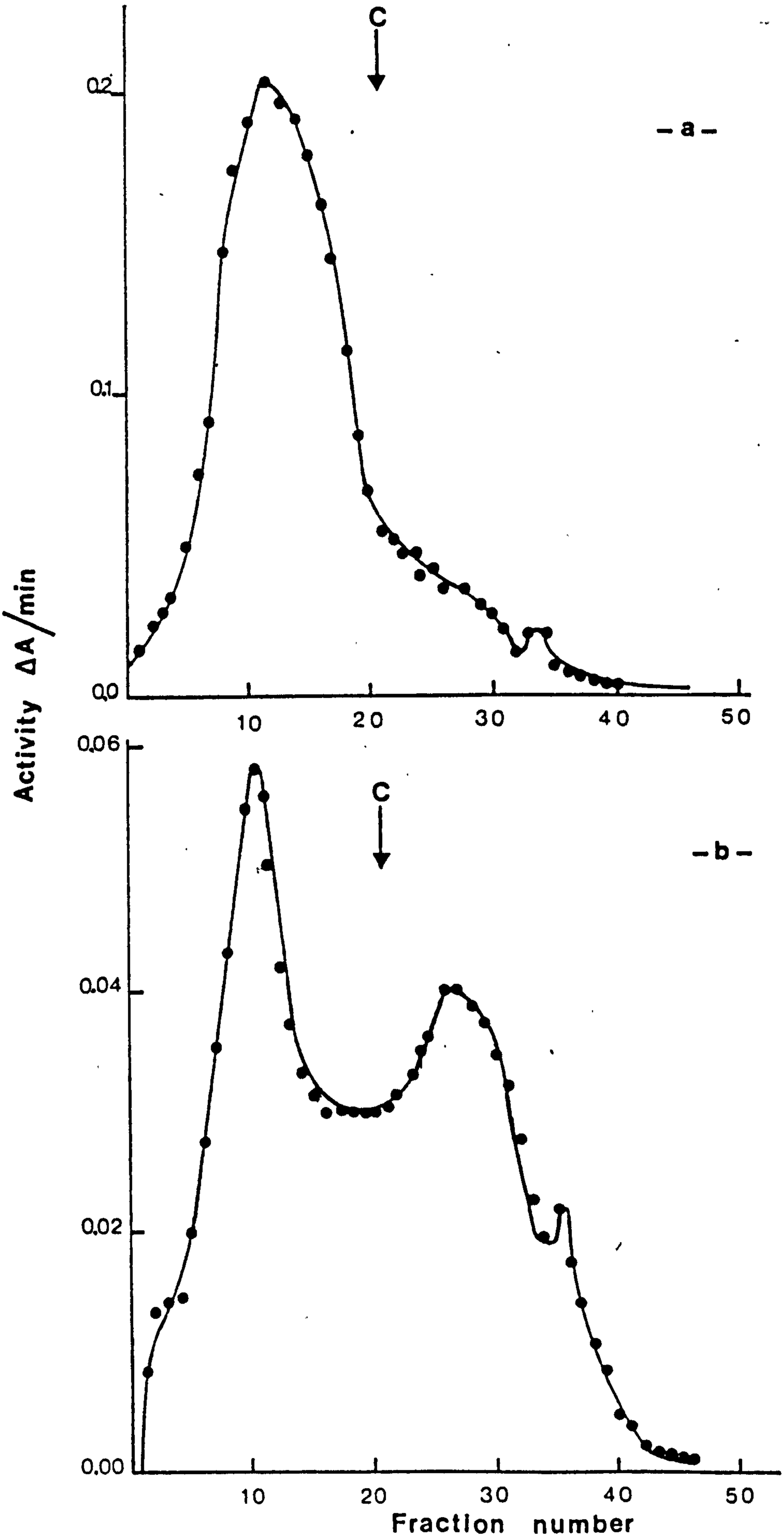
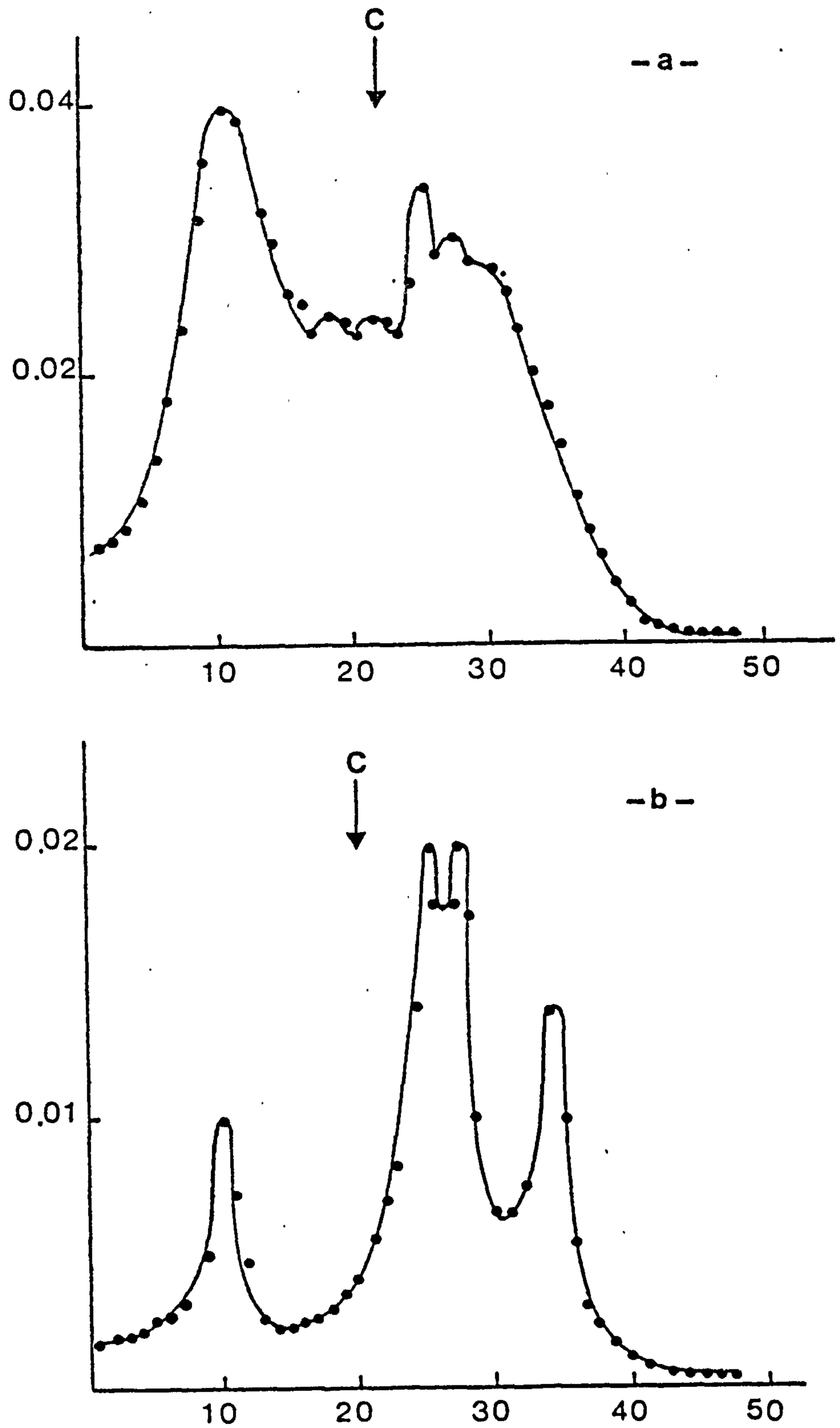


Fig. III.23
Sucrose gradient centrifugation: Triton-solubilized enzyme in the presence of antiproteases agents before (a) and after incubation at 37°C for 24 h.(b)



enzyme without previous concentration were applied on gradients. In Fig. III. 25a the elution profile of a sample from frozen rat brain can be observed. Only two peaks corresponding to sedimentation coefficients of 10S and 4. 8S were observed but a considerable proportion of enzyme was in a form with high molecular weight. The ratio was 20:66;14 for particulate enzyme, 10S and 4. 8S forms. In Fig. III. 26a another preparation obtained from identical procedure but from brain stored in deep-freeze for longer period of time than the previous, even the 16S form could be detected. The relative proportion for aggregated enzyme, 15. 2S, 10. 3S and 4. 2S was 3:12: 81:3.

When a sample from unfrozen brain was used, the obtained pattern is shown in Fig. III. 24b. Only two forms were measured with coefficients of 10S and 5. 2S in the proportion 85:15. It was not observed any particulate fraction.

f. Partially purified enzyme by affinity chromatography.

In these experiments samples of partially purified enzyme by MAP-agarose column were used.

(i) Enzyme without proteases treatment (Fig. III. 26b). Three forms with coefficients of 16. 5S, 9. 9S and 4. 1S in the ratio 2:95:3 were found.

(ii) Effect of proteases on partially purified enzyme .

Samples from purified enzyme were treated with trypsin, collagenase and papain, and incubated overnight at room temperature. The action of trypsin was checked above pH 5 and after the incubation period the enzyme was completely inactive.

Fig. III.24

Sucrose gradient centrifugation: (a) concentrated sample of Triton solubilized enzyme treated with NaCl (b) not concentrated sample of Triton-solubilized enzyme from fresh brain.

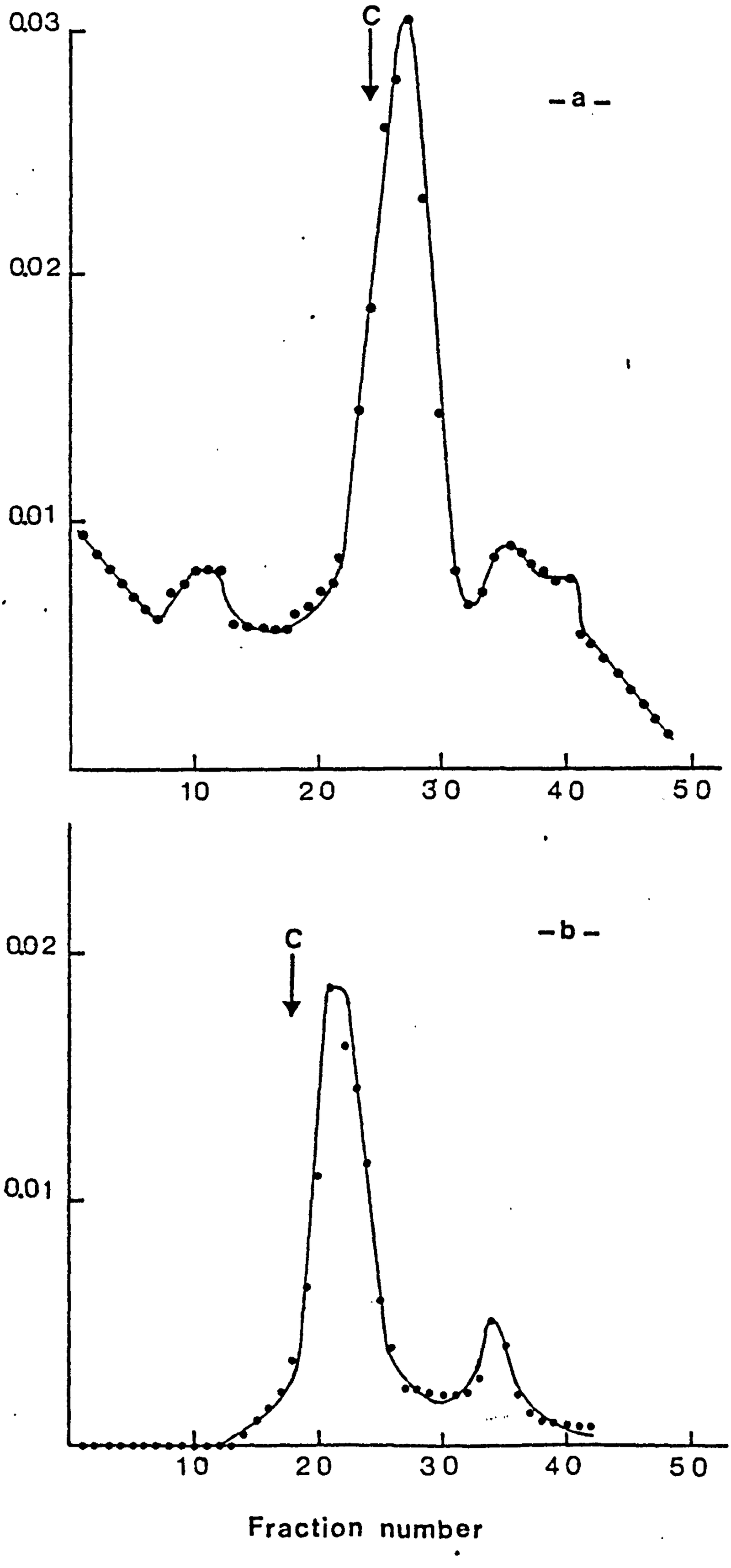


Fig. III.25
Sucrose gradient centrifugation: (a) Not concentrated and (b) concentrated samples of Triton-solubilized enzyme from frozen brain.

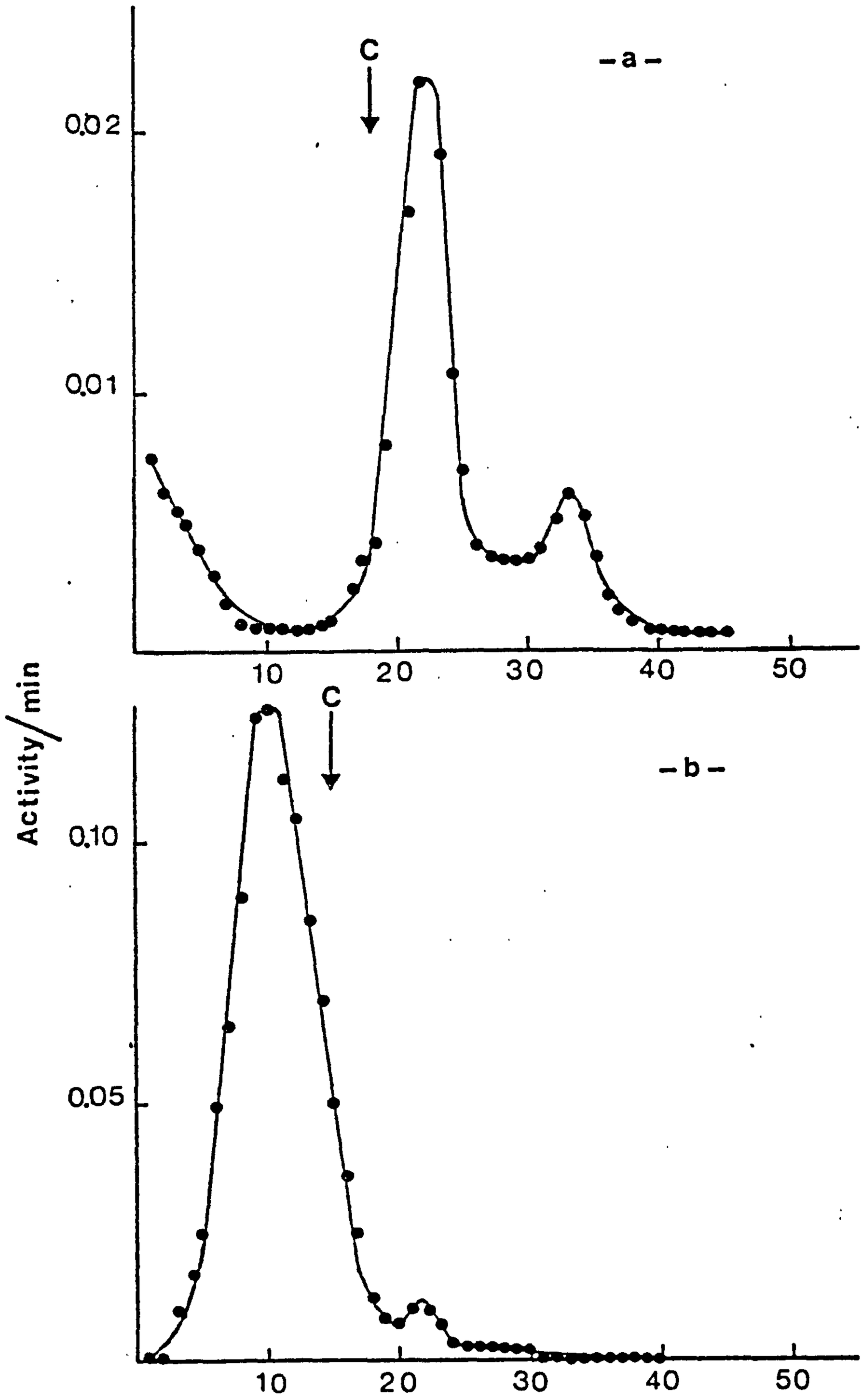


Fig. III.26
Sucrose gradient centrifugation: (a) Not-concentrated sample of Triton-solubilized enzyme from frozen brain, (b) partially purified enzyme by affinity chromatography.

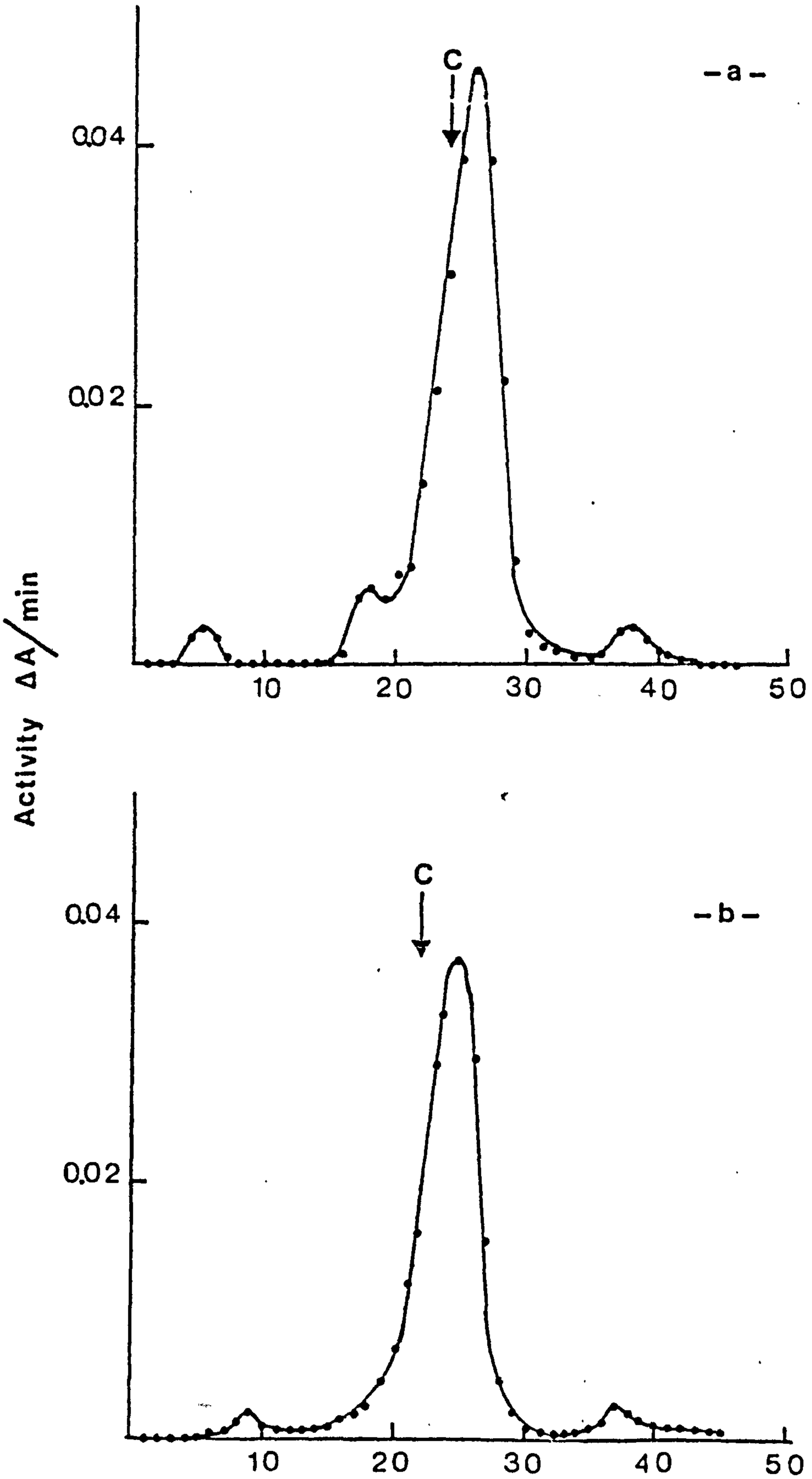
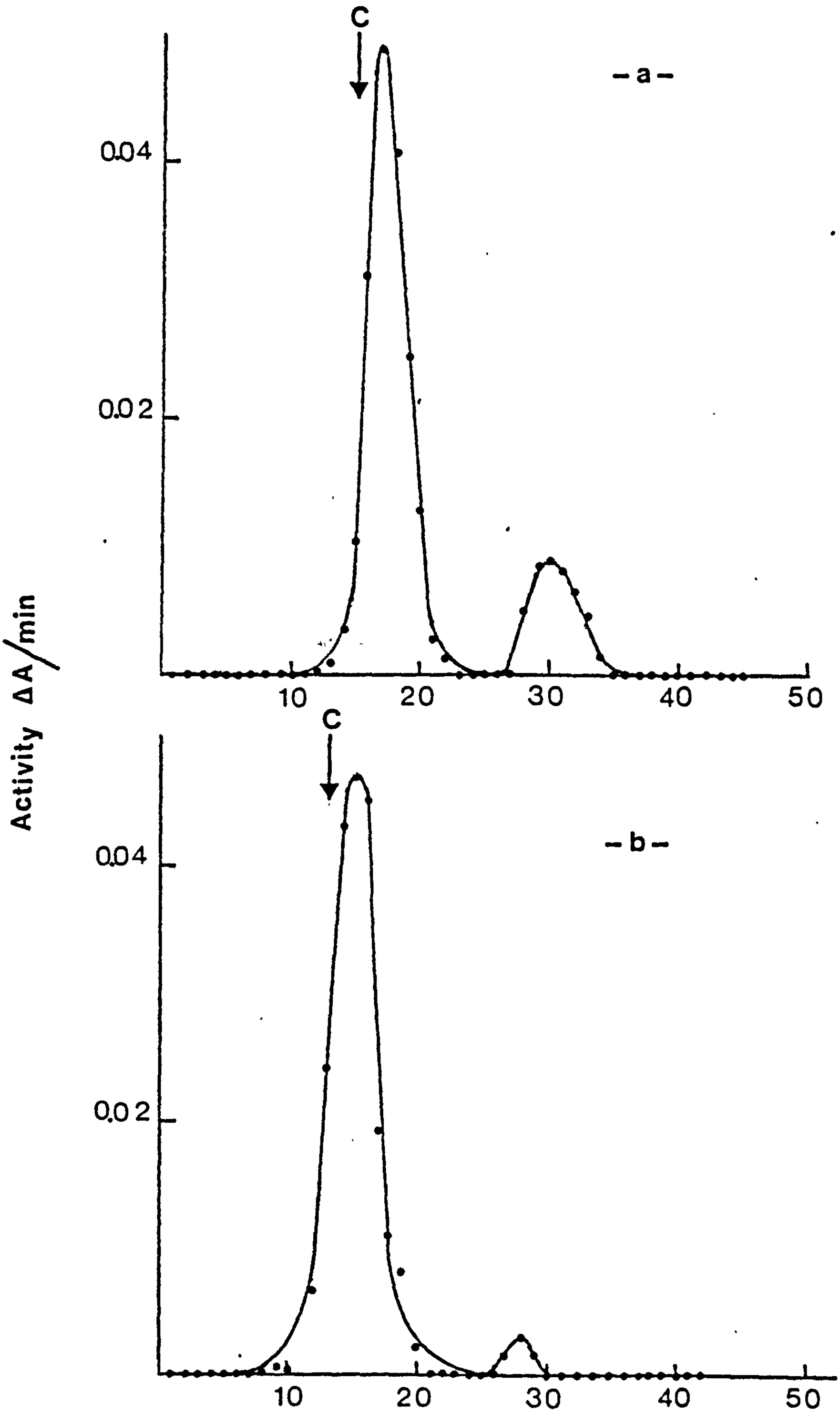


Fig. III.27
Sucrose gradient centrifugation: Partially purified enzyme by affinity chromatography treated (a) with papain (b) with collagenase.



Treated sample with papain gave the profile shown in Fig. III III.27a. Again two forms with sedimentation coefficient of 10.5S and 4.8S with areas in the proportion 81:19 and a very similar pattern with collagenase treated enzyme, but this time the areas ratio was 95:5 (Fig. III.27b).

## 2. Gradient Polyacrylamide Gel Electrophoresis in Slabs

Gradient Polyacrylamide Gel Electrophoresis also showed a multiplicity of bands, the number and molecular weight of which depended on the conditions.

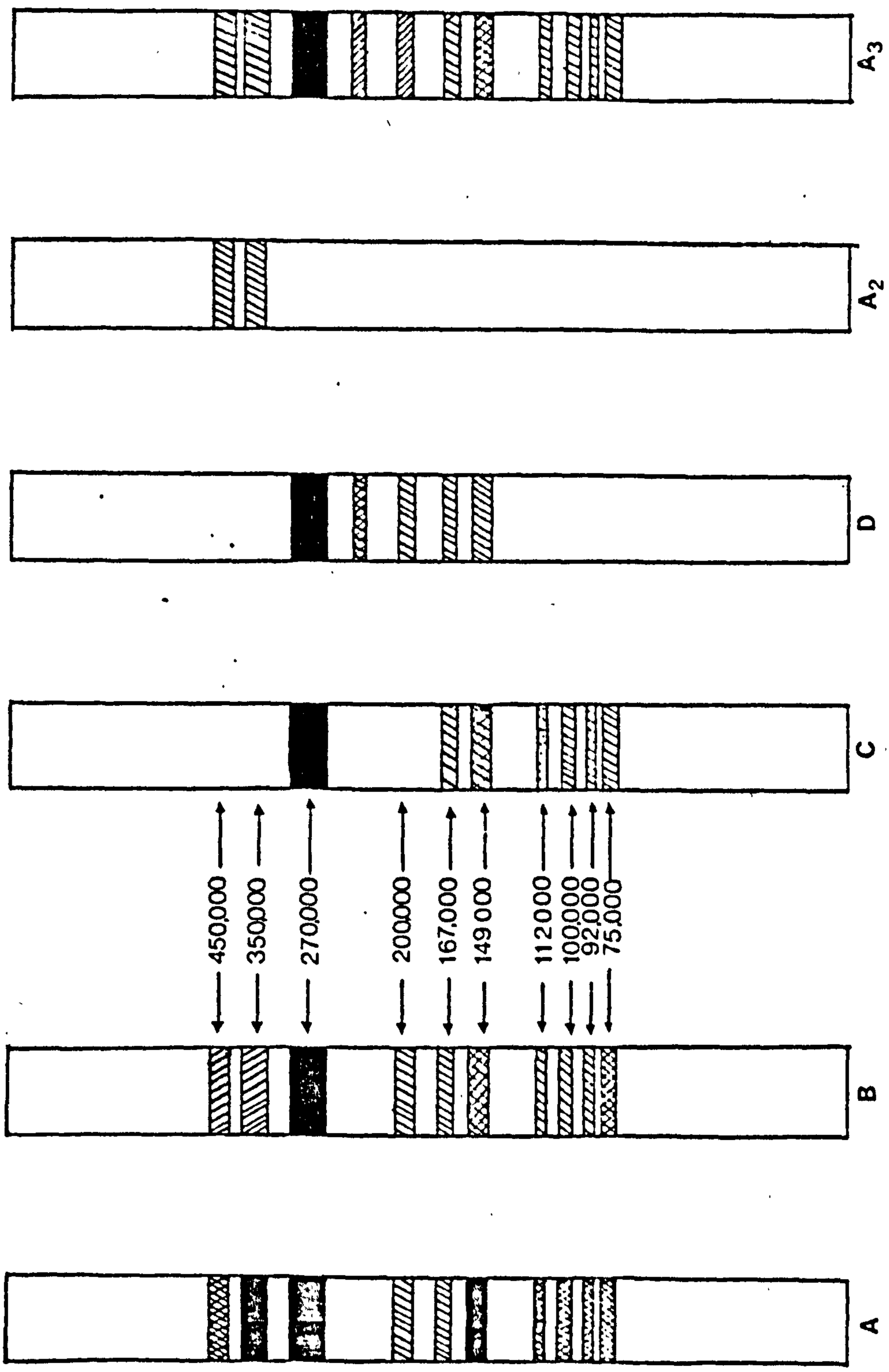
### a. Standard preparations and electrophoresis carried out in cold room

Assuming that the AChE molecules are spherical, then the molecular weights obtained for the "naturally soluble" fraction were 450,000, 350,000, 270,000, 200,000, 167,000, 150,000, 115,000, 100,000, 92,000 and 75,000 (Fig. III.28B). A very similar pattern was obtained with Triton solubilized enzyme (Fig. III.28A). Several bands were also detected with the toluene-stored brain. If the solubilization was performed just with buffer, bands with molecular weights of 270,000, 242,000, 167,000, and 150,000 (Fig. III.28D) were measured and when the enzyme was solubilized in the presence of Triton X-100 (1%) bands of 270,000, 167,000, 150,000, 100,000, 112,000, 92,000 and 75,000 were detected (Fig. III.28C)

### b. Electrophoresis performed at 37°C

Under these conditions bands of molecular weights of

Fig III.28
Gradient polyacrylamide-gel electrophoresis: A - Triton-solubilized and B - buffer-solubilized enzyme from frozen brain; C - Triton-solubilized and D - buffer-solubilized enzyme from toluene-stored brain. $A_2$ - Preincubation with ethopropazine. $A_3$ - Preincubation with eserine. Electrophoresis carried out in the cold room ($+4^{o}C$). Depth of staining was: ■ strong; ▨ moderate; ▧ light; ⁝⁝ faint.



420,000, 350,000, 260,000, 167,000, 150,000, 115,000, 105,000, 64,000, 50,000 and 40,000 were detected for "naturally soluble" and Triton solubilized enzyme from frozen rat brain (Fig. III.29 A', B'). Similar patterns were obtained from toluene-stored brain, buffer and Triton solubilized fractions but in those cases species with lower molecular weights of 64,000 were not found (Fig. III.29 C'.D').

i. Preincubation at 37°C for 24 h and electrophoresis carried out at 37°C. Not much difference with the previous experiment was observed, only that under these new conditions, species of higher molecular weight of 400,000 were detected in all preparations and species lower than 64,000 were only appreciated in Triton solubilized enzyme from fresh rat brain.

ii. Effect of Eserine and Ethopropazine on the patterns. Preincubation with eserine drastically diminished the number of bands. Only two faint bands were observed corresponding to the entities of molecular weights 410,000 and 350,000. However, preincubation with ethopropazine did not affect the patterns in any way (Fig. III.28 $A_2$,$A_3$).

c. Electrophoresis carried out at room temperature

The molecular weights of bands for the soluble enzyme were 550,000, 410,000, 340,000, 240,000, 170,000, 150,000, 100,000, 87,000, 83,000 and 75,000. Identical patterns were obtained for Triton solubilized enzyme from frozen rat brain.

d. Fractions from starch block electrophoresis

When the six bands separated by starch block electrophoresis from Triton solubilized enzyme were applied on the slabs, species of 780,000, 650,000, 500,000, 410,000, 340,000, 300,000, 220,000, 150,000, 92,000 and 75,000 were obtained from any starch block fraction..

e. Effect of proteolytic enzymes

The partially purified enzyme was treated with papain, collagenase and trypsin before applying to the polyacrylamide slabs. Species of 440,000, 350,000, 250,000, 200,000, 175,000, 150,000, 125,000, 110,000 and 92,000 were found in all cases but the band corresponding to 200,000 was very faint in the case of the papain treated enzyme. The collagenase treated enzyme gave very dense bands in the region 125,000-92,000.

f. Effect of Salts

When the Triton solubilized enzyme was treated with KCl, NaCl or $MgCl_2$ 0.2M bands of activity corresponding to species of molecular weight 500,000, 440,000, 350,000, 250,000, 150,000, 105,000, 92,000 and 76,000 were detected but if the salt concentration was increased to 2M the pattern was almost the same but the bands corresponding to 250,000 and 150,000 dissapeared completely (Fig. III.29$A_4$).

g. Effect of Mercaptoethanol

When the Triton solubilized enzyme was treated with 1%

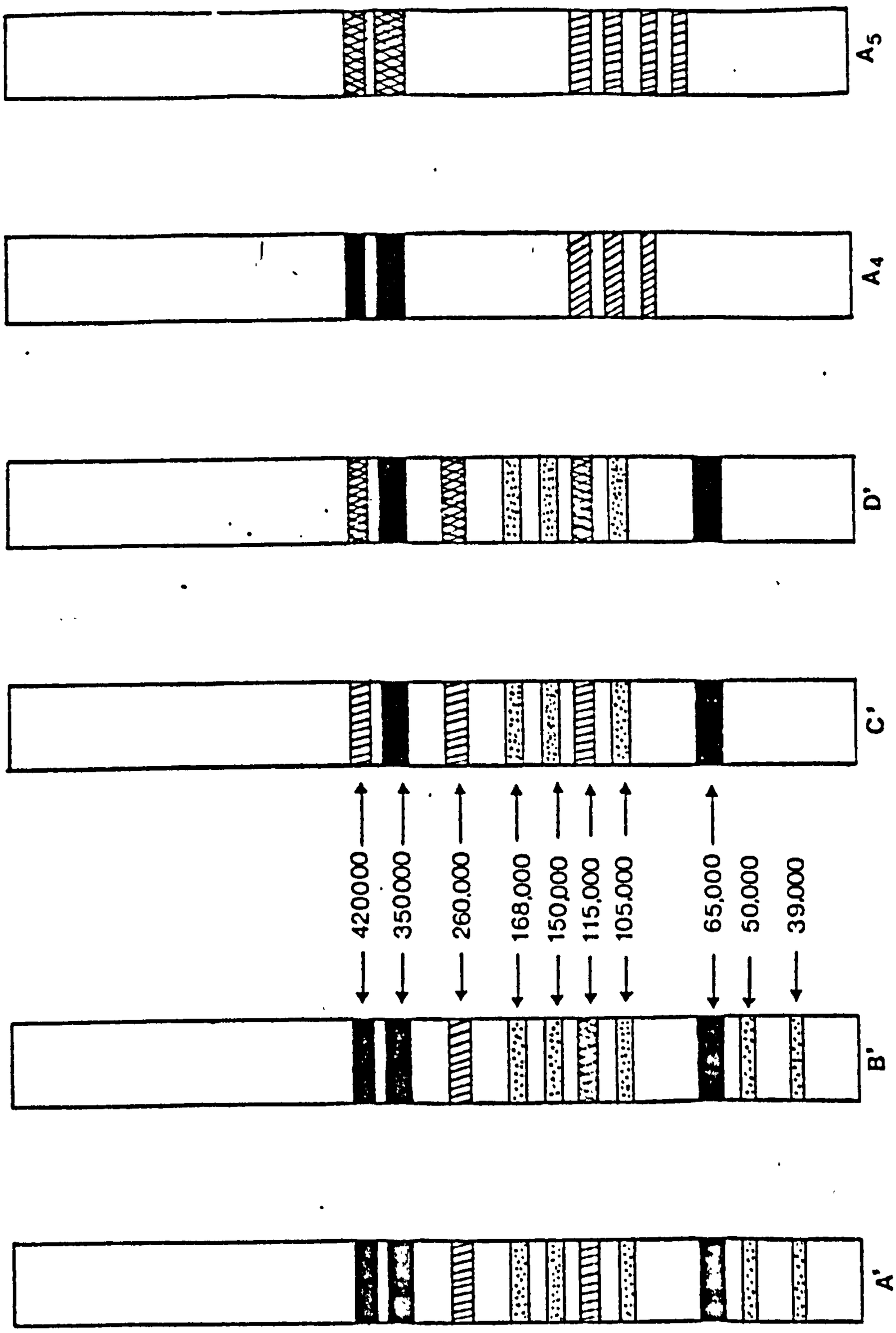


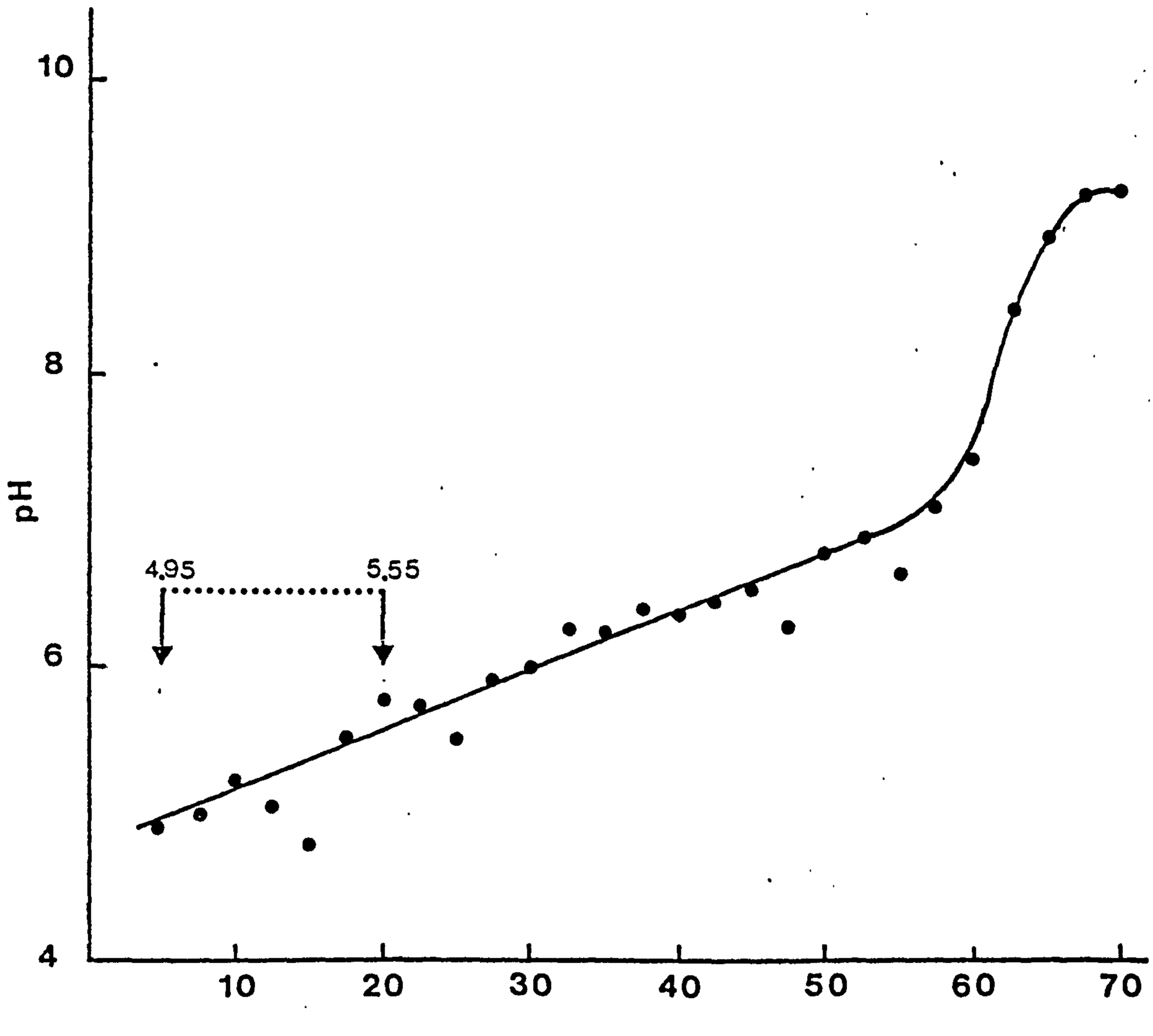

Fig. III.29
Gradient polyacrylamide-gel electrophoresis: A' - Triton-solubilized, B' - buffer' solubilized enzyme from frozen brain; C' - Triton-solubilized, D' - buffer' solubilized enzyme from toluene-stored brain $A_4$ - enzyme treated with KCl $A_5$ - enzyme treated with mercaptoethanol Electrophoresis for A' - D' carried out at 37°C Depth of staining was: ▩ strong; ▨ moderate; ▨ light; ▨ faint

mercaptoethanol, bands of molecular weights 410,000, 350,000, 120,000, 105,000, 92,000 and 76,000 were detected (Fig. III.29 A5).

## G. GEL ISOELECTROFOCUSING

When samples separated from starch block electrophoresis were applied to rods of polyacrylamide gel, containing "Ampholine" (pH 3.5-10) a quite broad band could be visualized after staining for protein. The stained protein was located quite close to the top of the gel, corresponding to a pH's interval of 4.95-5.55 (Fig. III.30). Iedentical result was obtained if the partially purified enzyme by affinity chromatography was applied.

FIG. III.30
Gel isoelectrofocusing of starch block fractions and partially purified enzyme (see text for details). Protein staining between the arrows.

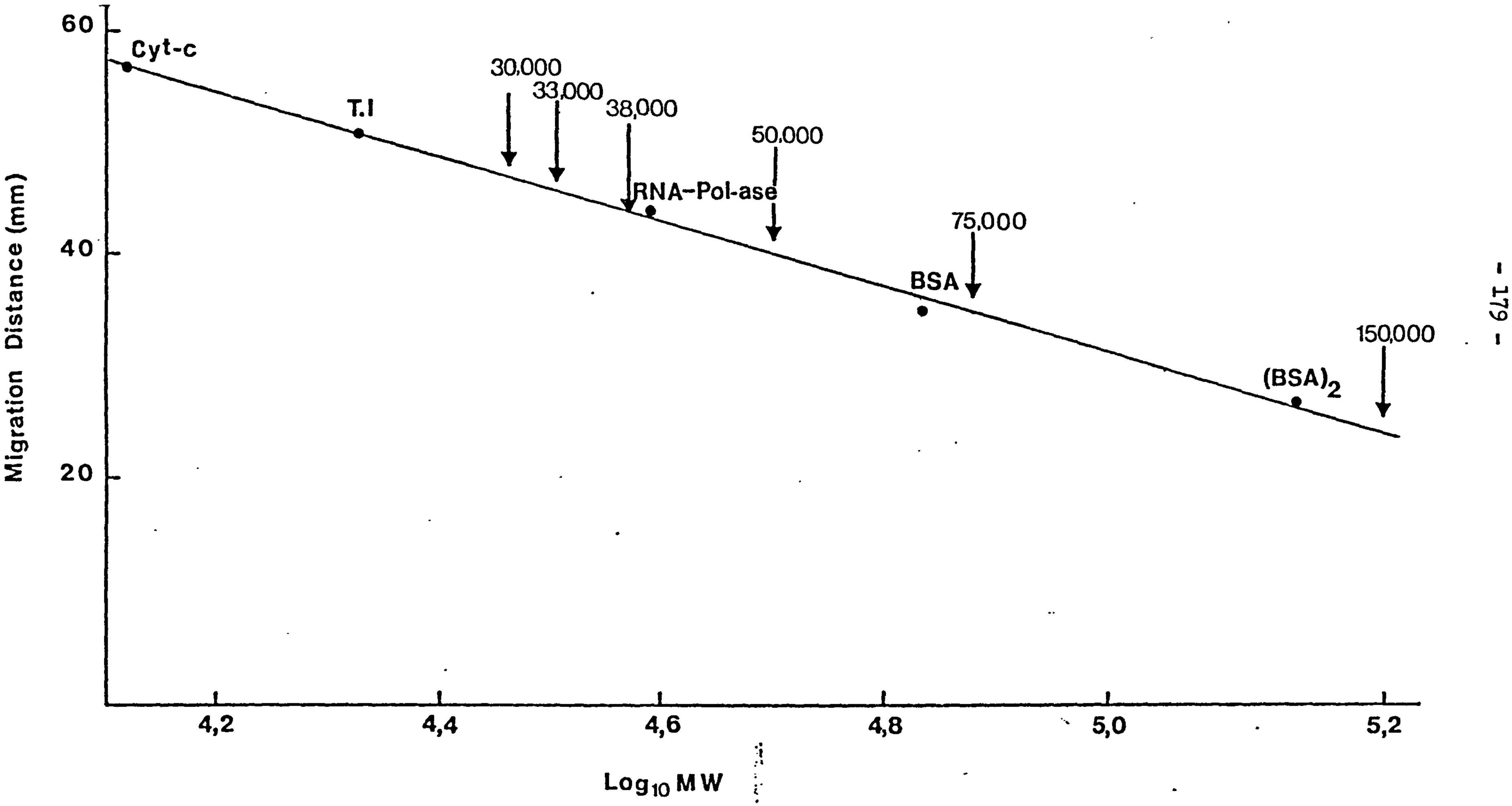


## H. SDS-GEL ELECTROPHORESIS.

The partially purified preparation was treated with mercaptoethanol and SDS, before applying to the slab gel. Staining for protein gave several bands with molecular weights of 150,000, 75,000, 50,000, 38,000, 33,000 and 30,000 (Fig. III.31).

Fig. III.31

SDS-Gel electrophoresis: Partially purified enzyme treated with SDS (1%) and mercaptoethanol (1%). The arrows show the positions of the protein bands. Standard proteins: Cytochrome-C (12.500); Trypsin-Inhibitor (21.500); RNA-Polymerase (39.000); BSA (68.000).



# SECTION IV

# DISCUSSION

## A. SOLUBILIZATION OF RAT BRAIN ACETYLCOLINESTERASE

In any study of enzyme solubilization an operational criterion must be established and in this investigation the enzyme was considered to be soluble if it remained in the supernatant fraction following centrifugation at 100,000 g for 1h. However, the presence of micromembrane fragments remaining in the supernatant cannot be discounted. In fact, Haaland et al (1971) demonstrated the presence of membranous vesicles by subjecting "solubilized" preparations to 200,000 g centrifugation followed by electron microscopy of the pellets. These had dimensions of 500-1000 Å.

### 1. Rat Brain Stored in Toluene

The use of organic solvents to solubilize membranes is based on their capacity to separate membrane lipids from protein. Their major drawback is that they may rapidly destroy enzyme activity and this was shown to be the case with brain tissue (Bernhein and Bernhein, 1936, Nachmansohn and Lederer, 1939 a, b).

#### a. Extraction with dilute buffer

Toluene treatment inactivated 50% of the enzyme but facilitated subsequent solubilization by dilute buffer (Table III.1). An increase of about 50% in the specific activity value for dilute buffer solubilized enzyme from toluene-treated brain with respect to the untreated enzyme was obtained. This increase was due to the fact that protein, in general, was very poorly solubilized whereas the enzyme reached an acceptable level of solubilization. During

the storage period, the formation of an exudate due probably to the mucin and lipid solubilization was observed. These results are in contrast with those of McIntosh (1973) who found that pig brain toluene treatment did not facilitate solubilization in dilute buffer. Toluene treatment of rat brain therefore appears to be more effective than the treatment of other mammalian brains but less efficient than with Electrophorus electroplax. (Rothenberg and Nachmansohn, 1947).

b. Detergent treatment

For proteins which are strongly associated with the lipid matrix of membranes the most useful method of extraction at present seems to be the use of detergents (Helenius and Simons, 1975). The solubilization of AChE is not an exception. Several groups of workers have successfully used detergents in the preparation of this enzyme (Ho and Ellman, 1969; Wright and Plummer, 1972; Adamson et al, 1977).

In these studies, the non-ionic detergent Triton X-100 was used following the method of Ho and Ellman (1969) with slight modifications. It can be seen from the results in Table III.1 that the use of Triton X-100 (1%) on toluene-treated brain was quite efficient in solubilizing the enzyme. A total of 58% of the homogenate activity was solubilized. However if the same procedure was applied to the untreated enzyme (Tables III.2, 3) it can be seen that the use of Triton on toluene-stored brain was not as effective as on the frozen brain. This was probably due to the nature of the acetonic powder-

like material. Because it was extremely compact, the approach (access) of the detergent to the membrane during the homogenization was greatly disminished. It is also possible that the toluene removes some non-polar lipids which could represent 'starting-points' for the breaking of the membrane by the detergent.

## 2. Frozen Rat Brain

### a. Dilute buffer

Incubation of rat brain homogenate in dilute buffer gave yields of between 10-13% of the total activity (Table III. 2). This figure agrees with the results of other workers who obtained very similar levels of AChE in this 'soluble' fraction (Ho and Ellman, 1969; McIntosh and Plummer, 1973; Devonshire, 1975; Plummer et al, 1975; Vigny et al, 1976). The specific activity value for this fraction was 0.036 μmol of acetylcholine/min/mg of protein and it was found to be similar to those reported by Reavill for the corr-esponding value of pig brain (0.028) and by Wenthold et al (1974) for EDTA solubilized enzyme from rat brain (0.040). It was thought possible that this soluble AChE might in fact be in equilibrium with the enzyme which was present on the membrane and not a true soluble fraction. However, when the 100,000 g pellet from this preparation was subsequently rehomogenized in buffer, very little AChE (4-5%) was brought into solution. This indicated that the AChE in the soluble fraction was a truly soluble pool of the enzyme, although its function is unknown. Probably this pool represents the "travelling" enzyme through the axons and also the

"waiting" for empty vesicles enzyme, but a satisfactory explanation so far has not been offered.

b. Autolysis

Because the AChE is membrane bound it would be reasonable to expect that proteolytic enzymes present in the brain homogenate, would, to a certain extent, digest the membrane components thus releasing the enzyme. About 15-20% of the enzyme bound to membrane, after removing the "naturally soluble" fraction, was solubilized after incubation for 16 h at 35°C, without inactivation of the particulate fraction. Although this was a considerable fraction of the total activity, it was not considered satisfactory to warrant further use. However, it could be interesting in the future to analyse the multiple forms in this fraction so as to compare them with the "naturally soluble" and "Triton solubilized" fractions.

c. Solubilization with Triton X-100

Homogenization of 100,000 g pellets with buffer containing Triton X-100 (1% w/v) solubilized the membrane-bound enzyme completely (Table III.2). The corresponding specific activity value was 0.33 μmol of acetylcholine hydrolyzed/min/mg protein or 4.30 μmol of acetylthiocholine hydrolyzed/min/mg protein. These results compare very favourably with those of Rieger et al (1976) who found solubilization up to 90% from rat brain by homogenization with Triton X-100 (1%) in saline buffer medium. The specific activity of such a preparation was 1.03 μmol AcTCh/min/mg. Using the same extraction medium, solubilization up to 90-95% was also measured

from rat muscle tissue with a specific activity of 0.14 μmol AcTCh/min/mg. About 65% of the enzyme present in membranous structure was solubilized by dilute buffer containing 1% Triton X-100 from pig brain (Plummer et al, 1975). The specific activity for that preparation was only 0.141 μmol ACh/min/mg protein. Up to 90% of the enzyme from chicken brain was brought into solution after extraction with saline buffer containing Triton X-100 (1%) (Vigny et al, 1976). The specific activity was as low as 0.28 μmol AcTCh/min/mg protein.

From these results it can be seen that rat brain is a quite important source of the enzyme, and in fact the AChE concentration is higher than in other mammalian or avian tissues.

Triton X-100 not only solubilized the enzyme satisfactorily but also produced an increase in the activity of the homogenate. About 120% with respect to the dilute buffer homogenate was measured (Table III.3). A number of reports have appeared in the literature on the effect of Triton X-100 on enzyme activity. Fiszer and De Robertis (1967) found no stimulation, Harwood and Hawthorne (1969) found considerable stimulation, while Gitler et al (1967) reported some inhibition of erythrocyte AChE at low Triton concentrations. Crone (1971) pointed out that the activation associated with solubilization depended very much on the method of preparation of the tissue fraction used as the source of the enzyme. A second factor accounting for the varied reports may be that the absolute Triton concentration has been quoted, rather than the detergent to tissue ratio. Crone (1971) found a constant stimulation of 116% for ratios of from 2 to 20 mg Triton/mg protein. No

stimulation was observed at ratios of from 0.075 to 0.6 mg Triton/ mg protein and intermediate ratios gave variable results. This increase in the activity of the homogenate is probably due to a more effective disruption of the membrane in the presence of Triton X-100 than is the case with dilute buffer.

d. The effect of proteases inhibitors

It has been suggested that endogenous proteases could affect the distribution of multiple forms of the enzyme (Silman et al, 1978 a, b; McLaughlin et al, 1976), so attempts were made to solubilize the enzyme in the presence of antiprotease agents. Under these conditions the yield of the "naturally soluble" form of the enzyme dropped to 4-5% and whereas the Triton solubilized fraction fell to 70-75% of the total homogenate. These lower values were due to an inactivation of 20-25% of the acetylcholinesterase activity. The inhibitor mixture included benzamidine and EGTA, which have been used in the isolation of procollagen where rigorous inhibition of proteolysis is needed to prevent its conversion to collagen (Byers et al, 1975), bacitracin, which prevented degradation of glucagon by microsomal extracts and leupeptin and pepstatin, two polypeptides, which have been shown to be very effective in blocking the action of lysosomal proteases from rat liver. The additional presence of an alkylating agent, N-ethylmaleimide suppresses proteolysis completely (Huisman et al, 1974; Dean et al, 1976). The inactivation of AChE could be due to any of these inhibitors but most likely arises from the strong alkylating action of N-ethylmaleimide.

e. Incubation with proteolytic enzymes

It has been reported by Hall and Kelly (1971) that incubation of intact muscle with proteolytic enzymes, such as trypsin and collagenase causes the detachment of active endplate acetylcholinesterase from muscle and 36% of the total activity was solubilized by collagenase treatment. The reaction takes place quite selectively because only the enzyme from the endplate region was released. Recently, Sketelj and Brzin (1977) have studied the effect of collagenase and papain on solubilization of AChE from the endplate region of mouse diaphragm. A considerable increase in apparent AChE activity was observed when proteolytic treatment preceded homogenization but this was not seen if the proteolytic enzymes were added to the homogenate. They explain their results in two different ways: a. AChE might be inactivated on homogenization while that inactivation could be preserved by prior solubilization with proteolytic enzymes, b. proteolytic treatment before homogenization might activate some "silent" AChE sites in motor endplates.

i Trypsin: Incubation of rat brain homogenate with trypsin for different periods of time and at two different temperatures had no effect on the enzyme either by solubilization or inactivation (Table III.1). This behaviour can be very easily explained by the fact that trypsin is rapidly hydrolyzed at pH values above 5. However experiments under that pH were not carried out because the enzyme ~~resulted~~ was quantitatively denatured at low pH. The method was therefore abandoned.

ii Collagenase: The effect of collagenase on acetylcholinesterase solubilization was also checked and the results were very disappointing since none of the enzyme was solubilized. However some inactivation of the enzyme was seen (Fig. III.1).

iii Papain: Finally, the action of papain on the solubilization of rat brain AChE was studied. In this case about 65-70% of the total enzyme activity bound to membranes was effectively solubilized. 10 $\mu$g of papain suspension was sufficient to solubilize the enzyme contained in 10 ml of a 10% (w/v) homogenate of rat brain, without appreciable denaturation. Papain is a proteolytic enzyme, composed of a single polypeptide chain, whose aminoacid sequence is known and with a molecular weight of near 21,000. This protease exhibits a very broad substrate specificity and contains in its structure a thiol group which is essential for its catalytic activity.

It is rather difficult to explain why papain is so good at removing the enzyme from the membrane. In fact the solubilization process seems to be more selective than the one with Triton because the specific activity for the papain preparation (0.446) was higher than the corresponding value for the Triton one (0.331). Generally speaking, the action of detergents is to release the enzyme through a real destruction of the membrane so that by this treatment, not only is the enzymatic protein released but also most of the protein components of the membrane. However in the case of papain, only those proteins which are exposed to this endopeptidase will be affected by this treatment. That is probably why papain solubilized less total protein than Triton. Finally we are faced with the

question as to whether the enzyme is a peripheral or an integral protein of the membrane. Singer (1974) has laid down several criteria for distinguishing peripheral from integral proteins. These include the requirement of detergents to dissociate them from the membrane and also the usually aggregated or insoluble state of the protein when in neutral aqueous buffer. Having stated his criteria, Singer also says that they must be applied with care, and he cites the example of AChE from bovine erythrocyte membrane which can be extracted by NaCl (1 mol/l) in the absence of divalent cations thus suggesting the possibility that AChE is a peripheral protein. Although he explains this by saying that membrane fragments enriched with AChE are probably released, the danger remains that liberal application of such criteria can lead to a misinterpretation of results. Before discussing how the brain enzyme may be attached to the membrane, let us have a look at the previous reports. It has been suggested by several authors (Silman et al, 1978 a,b ; Johnson et al, 1977) that the enzyme from the electric organ of the Electric eel is attached to the membrane via an elongated tail collagen-like structure. On the other hand, it has been reported by Hall and Kelly (1971) and Betz and Sakmann (1971) that collagenase releases the enzyme from muscle endplate with concomitant digestion on the basement membrane which contains polypeptides as major constituents (Kefalides, 1973). All these experiments have been carried out with tissues that are quite different from mammalian brain. Solubilization of calf brain without detergents has been reported by Hollunger (1973). When nearly 90% of the particulate

fraction was solubilized after three consecutive incubations in an ion-free medium containing low concentration of EDTA, similar results have been found by Adamson (1977) working on mouse brain AChE. Therefore it seems quite possible that the enzyme from mammalian brain is not very tightly bound to the membrane. The existence of a tail has not been reported in mammalian brain but if that is supposed in this tissue, the action of papain could be to digest the tail and thus detach the enzyme from the membrane but, if this is the case, why is collagenase so ineffective? There are several explanations for that question but it would be reasonable to think that if such a tail is present in the rat brain enzyme then it is not a collagen-like structure. Secondly, environment and steric effects might make the enzyme insensitive to the collagenase action. Thirdly, it may be that the actual rate of solubilizing the enzyme was smaller than the one of inactivation. Finally, the possibility that the brain enzyme is bound to the membrane in a different way from that which occurs in other sources, cannot be ruled out. Further experiments have to be carried out to confirm that the enzyme from brain is a peripheral protein and to establish the existence of a tail and its nature as a component of mammalian brain acetylcholinesterase.

## B. PURIFICATION BY AFFINITY CHROMATOGRAPHY

In order to study the properties of any biological molecule it is generally necessary to purify that molecule to a fairly high degree so as to exclude, as far as possible, any artefacts which could arise from the presence of impurities. In this department, pig brain AChE had previously been partially purified by a hydrophobic affinity column (McIntosh, 1973) and by three different types of affinity chromatography columns (Reavill, 1976). In this present study, two columns containing specific ligands for AChE were employed

### 1. MAC-Agarose Column

The MAC-Agarose column which was originally developed to purify the aggregating species of AChE from Electric eel proved to be very unsuccessful at obtaining a purified preparation from rat brain AChE. This was due to the fact that the enzyme was not retarded on the column, so this purification procedure was abandoned. This is in marked contrast to the excellent results obtained by Reavill and Plummer (1978) who purified the pig brain enzyme about 900 fold, with a yield of 44% and an activity of 148 μmol/min/mg. The reason for these contrasting results is not known but probably arises from differences in the tertiary structures of rat and pig brain acetylcholinesterases, in fact, the use of MAP-Agarose column was extremely inefficient at purifying pig brain enzyme whereas it was very successful at purifying the rat brain acetylcholinesterase.

## 2. MAP-Agarose Column

This affinity column was used previously by Goodkin and Howard (1974) to purify the enzyme from rat brain synaptosomal membranes and in the case of our preparation, 50 ml of Triton solubilized enzyme could be run through the column and 90% of the total activity remained bound to the MAP-Sepharose matrix. The enzyme was eluted with decamethonium bromide 50 mM and after amberlite treatment it was found to be 270 fold purer than the crude homogenate, with a 70% yield and an activity of 205 μmol acetylthiocholine hydrolyzed/min/mg protein. This compared very favourably with the results of Goodkin and Howard (1974) who purified the rat brain enzyme by 100-150 fold, and with those of Adamson (1977) who obtained a preparation from mouse rat brain with an activity of 87 μmol AcTCh hydrolyzed/min/mg protein a a purification factor of 1180 when a previously purified enzyme by gel-filtration on Sephadex G-200 was applied on the MAP-Agarose column. On the problem of eluting the enzyme from the column, it is convenient to note that Adamson, using a gradient of choline chloride obtained one single peak of activity at a concentration of inhibitor of about 40 mM. Reavill and Plummer (1978) reported that to elute the enzyme from pig brain, high concentrations of NaCl were also required, 100-350 mM. A good recovery was only obtained in our case if the inhibitor concentration was in the range of 40-50 mM. That could mean that the enzyme was quite strongly bound to the inhibitor N-methyl-3-aminopyridinium, in such a way that high concentrations of decamethonium were required to release the

enzyme. It seems reasonable to think that electrostatic and hydrophobic forces are implicated in the binding of the enzyme to the affinity column.

It was pointed out by Dawson and Crone (1974) that the more efficient the purification, the more difficult is the problem of removing the inhibitor from the purified product. This was exactly what happened in the case of rat brain AChE eluted with decamethonium bromide. The enzyme-decamethonium complex, after extensive dialysis against dilute buffer, or distilled water was found to be non-dialyzable and therefore because of the strong positive charge of the inhibitor, it should be bound to cation-exchange resins. So the efficiency of four of them at removing the inhibitor was studied. Dowex 50 x 4 inactivated the enzyme completely. This was probably due to the strength of the sulphonic acid residues of the resins that altered the structure of the enzyme. CM-Sephadex and CM-Cellulose did not inactivate the enzyme but neither did they remove the inhibitor. In this case, the carboxy-methyl groups were too weak to detach the decamethonium. However, Amberlite CG-120 proved to be extremely efficient at removing the inhibitor without inactivating the enzyme. Amberlite CG-120 has been extensively used in a radiochemical assay of AChE (Siakotos, 1969) to remove the non-reacting $^{14}$C-Acetylcholine chloride. The efficiency of this resin is probably due to two main factors, one could be the ability of the ionizable groups to bind selectively the inhibitor and the second, the size of the resin particle which could play a very important role in accommodating the complex prior to the subsequent removal of the polar inhibitor. The procedure is very rapid and it leads to

a partially purified enzyme without inhibitor which can be used for further kinetic studies. There is no precedent of such a method to remove inhibitors from enzyme preparations obtained by affinity chromatography and because most of the papers reported quite low yields, between 10-40%, it is possible that what they are getting is the enzyme bound to the inhibitors. Therefore, in subsequent studies it is advisable to treat the partially purified acetylcholinesterase from any source eluted by an inhibitor with Amberlite CG-120 to check if an inhibitor-free preparation has been achieved.

## C. STABILITY OF ENZYME PREPARATIONS

The preparation of partially purified enzyme was found to be very unstable when it was frozen but it was very stable after storage in the cold room (+4°C) for two-three weeks. These results agree fairly well with those reported by Dawson and Crone (1974) who found that the ox brain enzyme purified by affinity chromatography was quite stable when kept at 5°C for several weeks while the same preparation stored for 7 weeks in the deep-freeze lost 30% of its activity. Adamson (1977) also has reported that 75% of the activity was lost after freeze-drying a preparation from mouse brain that had been purified by affinity chromatography. To explain these results, it was decided to check the stability of different preparation after storage in a deep-freeze. The enzyme preparations from frozen or toluene stored rat brain were very stable at -20°C. However, in the presence of NaCl (final concentration 0.2M) that stability was diminished. After 3 days of storage the enzyme containing NaCl lost 50% of its activity. This denaturing effect was not observed with samples containing KCl, $CaCl_2$ or $MgCl_2$ (final concentration 0.2M). Probably this is due to a common phenomenon of the salting out of proteins but what is interesting to note is that the higher density of charge of $Na^+$ against $K^+$ is the determinant factor for the salting phenomenon. That could indicate that the enzyme is solubilized by Triton in a rather compact structure making the access of a quite big cation as $K^+$ is, very difficult. However, $Mg^{+2}$ and $Ca^{+2}$ had no effect on

stability probably because they bound preferently to some polar lipids contained in the brain preparation. When the enzyme solubilized by Triton X-100 was treated with Amberlite CG-120, it was more sensitive to the action of salts. While the preparation so treated was very stable during the storage in deep-freeze, the samples containing KCl or NaCl lost 50% of activity after three days of storage. That suggests that the amberlite treatment modifies to a certain extent the structure of the enzyme, making a more "open" tertiary or quaternary structure for acetylcholinesterase and therefore the access of $K^+$ to the aminoacids residues is facilitated. If the enzyme was firstly inactivated with decamethonium and secondly reactivated by amberlite treatment, the sensitivity to the ions was considerably increased. The Triton preparation submitted to this treatment, without addition of salt,was very unstable after storage in deep-freeze. In the presence of salts, $Na^+$ increased the instability considerably while $Mg^{+2}$ had very little effect on the stability of the enzyme preparations. The order of stability of the preparations containing different ions was as follows: $Mg^{+2} > Ca^{+2} > K^+ > Na^+ >$ just enzyme. It seems quite clear that the removing of inhibitor or, more probably, the binding of the enzyme to the inhibitor, decamethonium, modifies to some extent the structure of acetylcholinesterase. At this point it is worth remembering that the existence of peripheral anionic sites in acetylcholinesterase has been postulated (Changeux, 1966; Ronfogalis et al, 1972) and that most of these studies have been carried out on the nature of the binding between the enzyme and the depolarizing blocking agent

decamethonium. Supposing that the inhibitor-enzyme complex has a steric structure quite different from the enzyme itself, it would be possible that after the sudden removal of the inhibitor the modified structure of the enzyme could still be present and that this is more unstable than the native enzyme-structure. The $Na^+$ and $K^+$ ions might act as stabilizer of the enzyme restoring partially its former structure occupying the peripheral anionic site or neutralizing some negative charges of phosphate residues of the enzyme. Finally $Ca^{+2}$ and $Mg^{+2}$ mainly the second one would be very efficient at preventing this denaturation by binding to the peripheral anionic site or establishing inter- or intra-chain cross-linking, thus restoring the original structure of the enzyme. This is a rather speculative interpretation of the results on stability of enzyme but Hollunger and Niklasson (1973) reported that in the presence of $Na^+$ or $K^+$ ions, the solubilization of ox-brain was considerably diminished and the same phenomenon was observed if the solubilization was performed in the presence of $Ca^{+2}$ or $Mg^{+2}$. All these observations can be related to the work of Braun and Norman (1969) who found that high concentrations of monovalent cations ($K^+$, $Na^+$) could act as stabilizers of protein-lipid complexes by a common salt effect while the stabilizing effect of low concentrations of divalent cations could be due to inter- and intrachain cross-linking through protein carboxyl or lipid phosphate groups.

The partially purified enzyme showed the same stability on storage as the native enzyme. If the above interpretation is correct, the kinetic and thermodynamic properties of the native enzyme and

the amberlite-treated preparation containing $Mg^{+2}$ or $Ca^{+2}$ should be different. Here is a new branch of research with plenty of possibilities to understand a little more about the catalytic mechanism of acetylcholinesterases. Finally it would be convenient to comment briefly on the effect of ageing on samples stored in deep-freeze. Freshly prepared samples of enzyme from frozen or toluene-stored rat brain were perfectly clear, but after 5 or 6 months of storage, they became turbid. After centrifugation, the pellet contained some activity depending of the kind of sample; 25% and 11% for "naturally soluble" and "Triton solubilized" preparations from frozen brain and 11% and 3% for "buffer" and "Triton solubilized" enzyme from toluene-stored brain. Hollunger and Niklasson (1973) reported aggregation in different extracts of calf brain. They suggested that the aggregation of the enzyme might be a consequence of a transformation of the enzyme into an aggregating form by the action of a proteolytic enzyme or by a non-enzymic oxidation of SH-groups. They also showed that the aggregation was partially reversible by treatment of the aggregated enzyme with DEAE-Sephadex. Because of that, they claimed that a phospholipid (Grafius et al, 1971) or an aggregating protein, previously reported by Kremzner and Fei (1971) could be responsible for the aggregation.

From our own results, it seems quite clear that the aggregation is related to the amount of lipid and protein present in the different extracts. So, the enzyme from frozen rat brain solubilized with buffer, which has a higher content in protein than the Triton preparation,was aggregated to a greater extent than the "naturally

soluble" fraction, as it is shown by the measured activity in the pellet after recentrifugation (25% and 11% respectively). However assuming that the toluene, at least partially removes the lipids, the degree of aggregation resulting was diminished. The activity measured in the pellet was now 11% and 3% for buffer and Triton-solubilized preparations from toluene-stored brains. That could mean that the true determining factor for aggregation is some kind of hydrophobic protein-lipid interaction giving a network where the enzyme is included. The suggestion of an aggregating factor cannot be ruled out and in fact that might be the starting point for the interaction discussed above. However to establish a more convincing argument further experiments are needed.

Incubation of the four samples at 37°C for 24 h, had different effects on Triton solubilized samples and those extracted with dilute buffer. Activation in the range of 10-15% was found in the former samples and denaturation of 15-30% in the latter ones. Again, the formation of a Triton-lipid-protein complex seems to protect the enzyme against thermodynamic denaturation while the Triton-deficient samples were sensitive to heat and the toluene-stored sample with a low content of lipids was very easily inactivated.

## D. PROPERTIES OF THE ENZYME PREPARATIONS

### 1. Michaelis Constants and Optimum of Substrate

Membrane-bound AChE, buffer dilute and Triton solubilized enzyme either from frozen or toluene-stored rat brain showed inhibition by excess substrate, with the optimum in the range of 1-2 mM of acetylcholine chloride. There was no difference in Km values for particulate enzyme from frozen and toluene-stored rat brain (80 and 75 μM), the same happened with dilute buffer extracted enzyme (91 and 100 μM) although slightly different values were obtained for the Triton solubilized preparations (205 and 170 μM). The partially purified enzyme by affinity chromatography also showed inhibition by excess of substrate and the corresponding value for Km was 206 μM. The Km values found in the literature for mammalian brain AChE are 54 μM for mouse (Adamson et al, 1975); 230 μM for ox (Chan et al, 1972); 80 μM for pig (Plummer et al, 1975) and 420 μM for human brain (Patocka et al, 1969). The results for the rat brain enzyme all fall within this range. Very little difference was seen in the corresponding values for particulate and buffer solubilized enzyme, while the Triton preparations showed a lower enzyme-substrate affinity. It has been reported by Vigny et al (1978) that the catalytic activity of acetylcholinesterase from different tissues is not modified by the presence of detergent. Therefore the small change in Km value for Triton-containing preparation compared with the particulate fraction could be due to any kind of association between the enzyme molecules that could partially cover the active site, making the access of the substrate to that site more difficult.

## 2. Arrhenius Plots and Activation Energy

The Arrhenius plots of the membrane AChE from frozen rat brain did not show any break unlike the particulate fraction from toluene-stored brain which showed a break at about 25°C (Fig. III.7). The activation energy in the former case was 11.4 kJ/mol and for the the latter 28.4 and 12.9 k J/mol on either side of the transition temperature. The buffer solubilized preparations also showed a discontinuity in the Arrhenius plot with a transition temperature of about 25°C and activation energies of 11.6 and 4.9 kJ/mol for dilute buffer extract from frozen brain and 22.4 and 3.9 kJ/mol for the corresponding from toluene-stored brain (Fig. III.8). Finally the same behaviour was measured with Triton-containing preparation and values of 14.5 and 5.8 kJ/mol for Triton solubilized enzyme from frozen brains and 27.4 and 8.7 kJ/mol from toluene-stored brains were obtained (Fig. III.9).

It can be seen that except for the membrane preparation from frozen brain, the activation energy above the transition temperature (25°C) is in all cases much lower than that under 25°C. The enzyme thus works more efficiently at physiological temperature (37°C) than at low temperatures. The treatment with toluene almost doubled in all cases the activation energy with respect to the non-treated preparations under the transition temperature. This suggests that the toluene treatment modifies either the environment of the enzyme, its quaternary structure or its relative freedom in such a way that the polypeptide chains may be folded, increasing the activation energy. However, above the transition temperature there

was little change in the homologous values for the activation energies of frozen or toluene-stored brain, suggesting that a determined structure is favoured above 25°C. That configuration seems to be very efficient because the value for activation energy was as low as 4-5 kJ/mol. This suggestion could explain the absence of a break in the membrane preparation from frozen brains where the membrane itself might play an important rôle in maintaining the enzyme structure thus avoiding a conformational change. Ciliv and Ozand (1972) made studies on the Arrhenius plot of erythrocyte AChE and they found a phase change at 32°C with a soluble preparation. They suggested that the break was due to a change of the aggregation state of the enzyme or to a reversible change of enzyme protein between two states indicating that one of the states was more active at temperatures above 32°C. However, a satisfactory explanation for the existence of a discontinuity in Arrhenius plots with acetylcholinesterase from several tissues has so far not been found. It would be helpful to obtain the Arrhenius plot using enzyme purified by affinity chromatography because most of the lipid or protein interaction will hopefully have disappeared in such a preparation. Therefore the interpretation of those results will be much easier than the ones already commented on.

It has been demonstrated by Vigny et al (1978) that all forms of Electrophorus acetylcholinesterase possessed identical turnover numbers for active site and therefore quaternary interactions do not interfere with the catalytic efficiency of the active sites. This was also true for the 10S form, the particulate enzyme and the solubilized enzyme with or without detergent. They suggest that even in the case

of higher vertebrates where multiple molecular forms of AChE seem to represent physiological specializations of the enzyme, their structural differences are not correlated with any differences in catalytic efficiency. A plot of log $^{1}/Km$ versus $^{1}/T$ shows a linear correlation between Km values for the different preparations and temperatures. These results also support the above argument in that the different molecular forms each with a specific quaternary structure and with a particular behaviour against temperature were shown to have the same affinity for the substrate. Therefore the existence of different Km values for each multiple form can be ruled out.

## E. SEPARATION OF MULTIPLE MOLECULAR FORMS BY DIFFERENCES IN ELECTRICAL CHARGE

Maddy and Dunn (1973) have stressed their warning that as any protein which is extracted from its native membrane undergoes a change in the environment of that protein, all extraction methods must be suspected to at least some extent of producing aggregates which do not exist in the native membrane. Therefore, any solubilized fraction might conceivably be an aggregate of biologically dissimilar molecules of which only some might be responsible for the biological activity under study. It was therefore of great importance to bear these facts in mind in this work when drawing conclusions about the molecular weights of the various multiple molecular forms from rat brain acetylcholinesterase. In order to rule out the possibility of these anomalies, it was advisable to use several different extraction procedures and several more techniques for the characterisation and the separation of the molecular forms of the enzyme. Thus, if molecular forms which were prepared in different ways and resolved by different means appeared to be very similar, it would have been reasonable to assume that the different molecular forms were not artefacts of experimental procedure.

The three techniques of molecular separation used in this work - polyacrylamide gel electrophoresis, sucrose density gradient centrifugation and starch block electrophoresis - have all been used widely in biological investigation and have shown to given reproducible results.

## 1. Starch Block Electrophoresis

This technique, although rarely used nowadays, was employed for these studies so that any molecular forms which might have differed in charge could have been separated. Also, because it is a semi-preparative procedure, any separated enzyme can be further analysed.

Fig. III.11a, b, shows the profiles of the Triton solubilized enzyme from frozen and Toluene-stored rat brain using a 20 cm perspex former. In both extracts one major peak was resolved which was very slow-moving. The resolution was increased using a 30 cm perspex former and running the electrophoresis longer (Fig. III.13). Under these new conditions, the Triton solubilized enzyme could be resolved in 5-6 bands and because of their slow moving character, all of them were found close to each other. It was not possible to achieve a better resolution because the denaturation of enzyme was very high, in fact, the better the resolution achieved, by running the electrophoresis longer, the less the recovery. The "naturally soluble" fraction yielded only 2-3 bands, slow moving too, but the different profile obtained (Fig. III.14) does not mean that the distribution of species was essentially distinct from that of Triton solubilized preparation, because the recovery was different and the conditions were slightly different too. When the corresponding preparations from toluene-stored rat brain were analysed, very similar patterns to the Triton solubilized enzyme were obtained. 2-3 bands were seen in Triton solubilized fraction and almost the same pattern was obtained from buffer solubilized enzyme from toluene-stored

rat brain (Fig. III.13 and 14).

When the most active fraction from Triton-solubilized extract was reelectrophoresed, only one form was obtained with its corresponding position in between the markers.

Although the method is suitable for separation of species containing different net charge, it seems reasonable that there is a limit of resolution which depends upon the difference in electric charge of the species. Therefore, if that difference is very small true separation is practically impossible to achieve. This was so in our experiments (except in the case of Triton solubilized enzyme) where not only a separation of forms was obtained but a profile of bands with more or less resolution depending on factors such as current applied, time and the buffer, and even when the conditions were carefully controlled the technique was not very reliable. It has been reported by Wenthold et al, (1974) that AChE from rat brain exists in six forms, with isoelectric points of 5.51, 5.42, 5.30, 5.20, 5.10 and 5.04. That could be the explanation for the poor resolution achieved, because such a small difference in electric charge would not be high enough to separate these species completely.

## 2. Polyacrylamide Gel Electrophoresis in Rods

As a technique taken by itself Davis' (1964) disc electrophoresis method tells us little about the molecular weight of the different molecular species of proteins. However, it does offer a method of separating high molecular weights substances such as proteins and in this work of showing up differences in the molecular species prepared by different procedures.

The electrophoretic patterns of the acetylcholinesterase in this work is presented in Fig. III.15. The electrophoresis was carried out in cold room (+4°C) or in constant temperature room (+40°C) to see if there was any difference in the patterns under those two conditions. From frozen tissue, six rather intense bands and two faint ones where detected from Triton-solubilized enzyme whereas only four were detected in naturally-solubilized fraction. When the electrophoresis was carried out at 40°C the number of bands was reduced and now only four bands for Triton-solubilized and three strong bands and a faint one for buffer solubilized enzyme were seen. Similarly, from toluene-stored brain, four bands of activity were observed, one of which was quite faint and an almost identical pattern was detected at 40°C. On the other hand, the buffer preparation yielded three main bands and two faint ones at 4°C whereas at 40°C only one quite intense band and another faint one were obtained.

McIntosh and Plummer (1973), working on pig brain acetylcholinesterase, solubilized with different procedures, found some differences in the electrophoretic patterns, whatever the extraction medium used: the number of bands was always four but the mobility of the species did vary. Wenthold et al (1974) found that separation by isoelectric focusing of rat brain AChE species yielded different patterns according to the subcellular fraction studied. Their results showed that the number and intensity of bands from membranous structures enzyme were much higher than from the "soluble pool". They claimed that the released enzyme from the membrane might still carry some membranous material as carbohydrate attached

to the side chains of the protein molecule. This argument could explain too, the higher number of bands for the Triton-solubilized enzyme, where membranous material is detached from the membrane, with respect to the buffer solubilized enzyme. However, the toluene-treatment affects the membrane structure, and the enzyme could be released in a more uniform way, even after the use of Triton. However the possibility of inactivation of some specific form after toluene treatment cannot be ruled out. When the electrophoresis was performed at high temperature (40°C) the number and intensity of the bands was diminished. This effect could be due to thermal inactivation. Gel electrofocusing showed forms with isoelectric points in the range of 4.95 - 5.55, however the resolution was not very good because the pH gradient of "Ampholine" used (3.5 - 10) meant that most of the enzyme remained near the top of the gel. Further experiments using ampholines in the range of 3 - 6 should be made in order to obtain a much better resolution. Although this is not convincing, the result fits in quite well with that reported by Wrenthold et al (1974) who found five species with isoelectric points in the range of 5.04 - 5.51. The explanation of such a difference in charge has already been discussed.

## F. SEPARATION OF MULTIPLE MOLECULAR FORMS BY DIFFERENCE IN SIZE

### 1. Sucrose Gradient Centrifugation

#### a. Triton solubilized preparations

When an unfrozen Triton solubilized preparation of brain from a recently killed rat was applied on the gradient without prior concentration, two species with sedimentation coefficients of 10S and 5.2S, corresponding to approximate molecular weights of 200,000 and 75,000 were detected. No aggregated enzyme was found in this preparation. The ratio of areas for those forms was 85 : 15. If before the application of the same sample, the preparation was stored in deep-freezer (-20°C) for three days, two forms with sedimentation coefficients of 10S and 4.8S were observed but some aggregated enzyme was also detected. The ratio of areas was 20 : 66 : 14 suggesting that the aggregated material was produced at the expense of the 10S form. After several months of storage in the deep-freeze, some particulate enzyme and forms of 15.2S, 10.3S and 4.2S were detected. Their relative proportions were 3 : 12 : 81 : 3. However, if the sample was concentrated 20 times on an "Amicon Concentrator" before the application onto the sucrose gradient, forms of 16.0S, 10S, 6.6S and 5.2S were detected with the heaviest form accounting for more than 90% of the total activity. Several aspects should be noted from this first set of experiments. Rieger and Vigny (1976) reported the existence of two forms of acetylcholinesterase from rat brain with sedimentation coefficients

of approximately 10S and 4S. They claimed that the 4S is solubilized in absence of detergent and therefore they assumed that the light forms could be either light attached to the membrane or a soluble or degraded form of the enzyme. The heavy one was tightly bound to membrane and they suggested that this could be the functional form of acetylcholinesterase. They also showed that the older the animal, the higher is the proportion of the 10S form in such a way that after 14 days, the relative contributions of 10S and 4S forms in rat brain was 80-90 : 20-10. These results agree fairly well with those previously presented, where the contribution of the heavy 10S form is much higher than the 5S. The calculation of sedimentation coefficients for the lightest forms of the enzyme is extremely difficult because the volume of the drops eluted from the pierced tube vary slightly due to a hydrostatic difference of pressure from the start to the end and also to the decreasing density of the sucrose solution. Those differences have little or no effect on the heavy or medium forms but they are important in establishing the sedimentation coefficient for the lightest forms. From our experiments, when frozen fresh tissue or stored in deep-freeze, preparations, without prior concentrations, were analyzed by sucrose gradient centrifugation, there was very little difference in the patterns. Some particulate enzyme was present and also a species with high sedimentation coefficient (16S) but these were only minor components. These results are different to those reported by Silman et al (1978) who showed that in embryonic chick skeletal muscle preparation which had been frozen and thawed at least once, a new 13S form of the enzyme was detected which was not present in homogenates from fresh tissues. He

suggested that some autolysis had taken place which could explain the appearance of this anomalous form at the expense of a 6.5S which normally is found in that tissue. However in a later paper (Silman et al, 1978) he found that by storing the homogenates from chick muscles at -20°C or by freezing the tissue prior to homogenization, there was a considerable decrease in the contribution of the heavy form (19S) while the lighter forms became more dominant (4.5S and 6.5S). Therefore it seems clear that autolysis had taken place on those extracts, but a similar process was not found, for rat brain preparation, providing the samples were not concentrated.

b. Concentrated standard preparations

Frozen samples of Triton and dilute buffer solubilized enzyme from frozen and toluene-stored rat brain were concentrated on an "Amicon Concentrator" before being applied to the gradients. Forms with sedimentation coefficients and approximate molecular weights of 14.5-15.2S (370,000-350,000), 10.1-10.3S (220,000-200,000) 8.8-7.8S (165,000-135,000) and 6.5-5.5S (98,000-75,000) were found in all cases, but their relative contribution was quite different, 50 : 25 : 22 : 2 and 78 : 5 : 15 : 2 for naturally and Triton solubilized enzyme and 68 : 5 : 25 : 2 and 63 : 12 : 23 : 2 for dilute buffer and Triton solubilized from toluene-stored brain. Previous analysis of the multiple molecular forms of acetylcholinesterase from mammalian and avian tissues by sucrose gradient centrifugation have been reported by several authors. The results reported by Rieger and Vigny (1976) on rat brain have already been commented on but they also worked with rat diaphragm muscle and found an

additional form of the enzyme with a sedimentation coefficient of 16S, which had previously been characterized by Hall (1973) who showed that this form could only be solubilized with detergent. They also reported that the 10S form was transformed into the 4S when the preparation was stored for several days at room temperature. They did not find the 16S form in brain and suggested that this form was perhaps not sufficiently solubilized under the conditions of homogenization. From the Stokes radii obtained by gel filtration, they calculated molecular weights of 115,000 and 435,000 for the 4S and 10S forms respectively. They compared these forms with those of a similar size found in electric eel and suggested that the tail structure is not present in the rat brain enzyme. In a later paper on rat muscle AChE, Vigny's team (1976) reported that the 16S form was never detected in smooth muscle extracts and that it disappears after denervation but after re-innervation the 16S form reappeared in the zones where nerve and muscle were in contact. From these experiments, they concluded that the 16S form is specific for endplate acetylcholinesterase and an excellent biochemical marker of the neuromuscular junction. Recently Di Giamberdino and Conraud (1978) carried out experiments on sciatic nerves of rat and found several forms with sedimentation coefficients of 16.5S, 10S, 6.5S and 4S in the relative proportion of 2 : 63 : 7 : 28. They claimed that because they had used a SW41 Beckman rotor, the 16.5S and 6.5S which were not seen before could now be detected. Studies on pig brain (Plummer et al, 1975) have shown the existence of one species with a coefficient of ~11S from Triton solubilized enzyme

and two additional forms of about 15S and 19S from just buffer or EDTA containing buffer preparations. They did not find smaller forms in that tissue. From chick brain, forms with coefficients of 11S, 6.5S and 4S have been shown by Marchand and Chapouthier (1977) while Vigny et al (1976) reported the presence of 11S and 6.5S (ratio 75 : 25) and 11S, 6.5S and 4S from brain and peripheral or sciatic nerves respectively in chicken. Silman et al (1978) have found that both autolysis and tryptic digestion produced conversion of the 6.5S into the 13S. From the Stokes radii they calculated molecular weights of about 260,000 and 365,000 for the 6.5S and 13S respectively, the latter being more globular than the 6.5S form.

Summarising the above review, we can conclude that in mammalian brain, the 10S species is the major form while the 4S form is only sometimes detected as a minor component. The 10S form may be converted to an aggregated 16S form, and in this transformation autolysis followed by electrostatic and/or hydrophobic interactions play an important rôle and indeed, aggregation of AChE has been reported by several research workers (Rieger et al, 1972; Dudai et al, 1973). Hollunger et al (1973) even suggested the possibility of a specific factor responsible for this aggregation. They suggested that such a factor might be a phospholipid (Grafius et al, 1971) or the aggregating protein whose existence was reported by Kremzner and Fei (1971). Finally, the possibility that the solubilization procedure has increased the tendency of the enzyme to aggregate (Maddy and Dunn, 1973) cannot be ruled out. Therefore the 15-16S form in rat brain as the 13S in chick brain could be an

autolyzed and aggregated form obtained from the 10S and 11S forms from rat and chicken brain respectively. Probably also, the 15S form of brain had a molecular weight higher than the calculated one from the Martin and Ames relationship (1961) because on polyacrylamide gradient gel electrophoresis forms with MW greater than 450,000 were detected from samples which were stored in deep-freeze for more than 6 months. The 10S form could represent a subunit dimer with or without an additional component and with a molecular weight in the range of 200,000 - 220,000. After concentration of the samples (either Triton or just buffer solubilized) an additional form with sedimentation coefficient of about 8S and molecular weight of 150,000 was also detected. Its contribution was very important in the buffer solubilized samples and in Triton solubilized enzyme from toluene-stored brain. The 10S + 8S activity in the concentrated four standard preparations were 47%; 20%; 30% and 35%, values so close as to think that 8S form might be a degradative form of the 10S, the small difference in sedimentation coefficient suggests the removal of a relatively light component and therefore 10S could be a dimer structure where the subunits were attached to a component which on removal could give the 8S form. Further experiments should be carried out to provide stronger support for that argument.

c. Effect of temperature and salts on concentrated samples

Preincubation of concentrated samples from frozen brain at 37°C had a truly surprising effect on the distribution patterns in both dilute buffer and Triton solubilized preparations. Forms with

sedimentation coefficients of 16S, 10S, 7S and 5S were detected as previously, but their distribution was 13 : 43 : 24 : 19 and 5 : 80 : 5 : 9 for dilute buffer and Triton solubilized preparation respectively. The activity of 10S + 7S was also constant as before. A comparison of the relative areas with the non-preincubated samples showed a major contribution of the 10S form compared with the 15-16S species. In fact, it appeared that the 16S form was almost completely inactivated leaving the other forms unchanged. The same phenomenon was observed when NaCl was added to a concentrated sample to a final concentration of 0.2M. The distribution in this case was 15 : 65 : 11 : 9. From these results we can conclude that the 15-16S form of the enzyme is extraordinarily sensitive to heat and salt effects. That could be the reason why other investigators were unable to detect that form in previous studies where they extracted the enzyme in saline buffer where the concentration of NaCl could be as high as 1 M. Further experiments will be carried out with saline buffer to see if the 16S form (with and without concentration) is still present. High salt concentration and heat are denaturating agents and can thus modify the quaternary or tertiary structure of the enzyme. However it should be noted that the other forms did not show this behaviour and as mentioned previously, it seems reasonable to think that hydrophobic and electrostatic interactions could play a very important role in the formation of the 16S form.

d. Effect of antiproteases agents on concentrated samples

When the solubilization of the enzyme was performed with buffer containing antiproteases agents and Triton X-100, the

forms detected on sucrose gradient centrifugation of the concentrated samples were 16S, 10S, 7-8S and 4-5S, in the ratio 45 : 46 : 4 : 1, but after incubation of the concentrated samples at 37°C for 24 h, the distribution was 20 : 35 : 30 : 15 and as before, the 16S was almost completely inactivated while the total 10S + 8S activity remained practically constant under both situations. Because the homogenization medium contained antiproteases agents (see before), the results suggest that the inactivation of the 16S form does not arise from proteolysis but rather hydrophobic or electrostatic interactions may be implicated in that transformation. However, unlike the 16S species, the lightest form of enzyme could be subject to autolysis since its contribution increased substantially in the presence of antiproteases. Studies with the same antiprotease medium have been carried out by Silman et al (1978) using chicken or rat muscle as the source of the enzyme. They showed that in the presence of antiproteases agents, the chick muscle preparation presented two major components (19S and 11S) and two minor ones (15.7S and 6.5S) while the 4.5S was present in homogenates without antiprotease agents. Also, in the absence of antiproteases the contributions of the heavier forms (19S) decreased and the lighter forms accounted for a greater part of the activity. The same study on rat muscle showed that if the tissue was homogenized in the absence of antiproteases inhibitors the 16S form was a minor component compared with the 10S and 5S but in the presence of antiproteases agents the 16S was the dominant form. They argued that the antiproteases prevented degradative phenomenon and as a result of that, the distribution of molecular forms was much simpler than it was previously suggested.

Their results however do not agree with ours because with brain homogenized in the presence of antiproteases, the 10S increased with respect to the non-antiprotease medium preparation. It could be that the antiprotease medium prevented autolysis and aggregation of the 10S. However after incubation at 37°C, the 16S form was almost completely inactivated, while the 5S form was notably increased. That 4.5S form seems to be the smallest active form and with a molecular weight in the range of 75,000-100,000 could be the monomeric structure of acetylcholinesterase in all the tissues so far studied.

e. Effect of proteolytic enzymes on partially purified enzyme

When the partially purified enzyme by affinity chromatography was applied onto the gradients, one major form (10S, 95%) and two minor forms (16.5S and 4-5S) were detected. While incubation with collagenase had no effect on the pattern, papain increased the proportion of the lightest form, representing after the proteolytic treatment about 20%. Therefore papain seems to detach the possible monomeric structure from the suggested dimeric one, 10S.

As final conclusion it can be said that the 10S is the dominant form of rat brain acetylcholinesterase "in vitro", while the 15-16S seems to be an associated form but if that 16S form actually exists in the membrane, "in vivo" remains at this stage of the work, unknown. Further investigation is needed to answer this important question to understand the physiological aspects of this striking enzyme.

## 2. Electrophoresis in Gradient Polyacrylamide Slabs

Electrophoresis on gel gradients when run to equilibrium has the advantage over the disc electrophoresis method in that molecules almost separate according to differences in their molecular size thus allowing us to estimate the molecular weights of the multiples molecular forms. It also means that the values of the molecular weights can be compared with those ~~attained~~ obtained by sucrose density gradient centrifugation.

The principle involved with this technique is that proteins pass through a gradient of progressively smaller pores in the polyacrylamide until they reach the point where the size of pore restricts their passage. Here the proteins concentrate and can be detected by specific staining. A great advantage of the technique is that several proteins can be run side by side on the same gel, thus allowing the direct comparison of the proteins. This also removes any inaccuracies caused by differences between individual gels. The band patterns obtained from the standard proteins, showed a straight line relationship when migration distances were plotted against the $\log_{10}$ of molecular weight (Fig. II. 3) (McIntosh, 1973; Reavill, 1976).

### a. Standard preparations

Assuming that the AChE molecules are spherical and that there is a linearity between migration distances, and log of MW, the forms obtained in the four preparations on electrophoresis could be classified into three groups: Heavy forms (440,000, 350,000 and 270,000); medium size forms (200,000, 170,000 and 150,000) and light forms (116,000, 105,000, 92,000 and 75,000). All of them were

found in naturally soluble and Triton solubilized enzyme from frozen brains (Fig. III.28). However, the "naturally soluble" enzyme from toluene stored brain showed an additonal band of 240,000 and the absence of forms smaller than 150,000 and greater than 270,000. The Triton solubilized enzyme from toluene-stored brain showed the presence of the lighter forms (Fig. III.28). The papain solubilized enzyme and the Triton extract of brain prepared in the presence of antiproteases did not show any difference with the corresponding Triton solubilized enzyme in non-antiproteases medium. Very little change was observed from the patterns of partially purified enzyme by affinity chromatography and the one treated with collagenase or papain. The only difference under these two situations was a higher intensity of staining in the region 125,000-92,000 for the collagenase-treated preparation. However, if the electrophoresis was carried out at high temperature (37°C) or if the sample was pre-incubated at 37°C for 24 h prior to the electrophoresis, additional bands with molecular weights of 63,000, 50,000 and 39,000 were detected. Preincubation of the slabs with eserine and ethopropazine confirmed the acetylcholinsterase nature of the bands. The isolated fractions by starch block electrophoresis showed the above general sequence of bands but also forms with very high molecular were visible (800,000, 650,000 and 500,000).

b. The action of mercaptoethanol and salts on the Triton solubilized enzyme pattern

The addition of salt to a final concentration of 0.2M had no effect on the patterns but if that concentration increased to 2M the

150,000 daltons form completely disappeared. Mercaptoethanol drastically reduced the number of bands and only lighter and heavier forms were detected. A full range of molecular weights have been reported in the literature for multiple forms of mammalian nervous tissue.(Table IV-1). The values show differences using the same source of enzyme and different estimation technique or even with the identical experimental procedure. On the other hand, it seems possible that the solubilization procedure has a very important effect on the calculated molecular weights and with these points in mind, I shall discuss the above results.

A common feature in the whole set of samples analysed was that the bands appearing in the three groups already mentioned are present under most of the conditions of assay. The lighter forms were always four species with molecular weights of 75,000, 92,000, 100,000 and 115,000. The separation between them was as little as 1-2 mm, but even so, they could be clearly visualized. It seems reasonable to consider that the lighter forms would present a common sedimentation coefficient of about 4-5S. This argument is strongly supported by the results of Rieger and Vigny (1976) who assigned a molecular weight of 115,000 for the 4S form of rat brain acetylcholinesterase. Adamson (1977) found a predominant form (70-80%) in mouse brain or muscle extracts with a molecular weight of 74,000 as estimated by gel filtration, while Hollunger and Niklasson (1973) estimated a molecular weight of 80,000 for the monomer of calf brain enzyme. The next question to answer is why four different weights as close as 75,000, 92,000, 100,000 and 115,000? or in other words,

TABLE IV-1

Molecular Forms of Mammalian AChE as Determined After Various Types of Solubilization

| Source | Method of solubilization | Mol. Weights | References |
|---|---|---|---|
| Rabbit sciatic nerve | Triton X-100 | 170,000-310,000 | Skangiel-Kramska and Niemerko, 1971 |
| Calf candate nucleus | Butanol | 284,000-360,000<br>161,000-205,000 | Jackson and Aprison, 1966 |
| Bovine candate nucleus | Repeated homogenization with EDTA | 395,000-270,000<br>130,000 | Shirachi et al., 1972 |
| Bovine candate nucleus | 0.32M - sucrose<br>1 mM - EDTA | 1 million-500,000 | Hollunger and Niklasson, 1973 |
| Bovine brain | 0.32M - sucrose<br>1 mM - EDTA | 390,000-270,000<br>130,000 | Chan et al., 1972 |
| Pig brain | Triton X-100 | 350,000-266,000<br>198,000-130,000-<br>60,000 | McIntosh and Plummer, 1973 |
| Rat brain | Triton X-100<br>bacterial protease | over 200,000<br>100,000 | Crone 1971<br>Ho and Ellman, 1969 |
| Rat brain | 0.32M - sucrose<br>1 mM - EDTA | 650,000-500,000<br>320,000-150,000 | Wenthold et al., 1974 |
| Rat brain | Triton X-100<br>1 M - NaCl | 435,000-115,000 | Rieger and Vigny, 1976 |
| Mouse brain | 0.32M - sucrose<br>1 mM - EDTA | 80,000 | Adamson, 1977 |

are these forms different monomeric structures? The answer is rather difficult. The existence of four different monomeric structures of the brain enzyme would seem unlikely but on the other hand because the same phenomenon was observed in the naturally soluble enzyme preparation, the possibility of an artifact due to the use of Triton has to be ruled out. The following interpretation might be suitable to explain the multiplicity of these light forms. If as a result of the homogenization procedure, a single molecular species is released from the soluble or membranous pool, carrying a range of charges, it seems probable that these charge variants will migrate to similar but not identical positions in the slabs because of the difference in their electric charge. If that is true, the real molecular weight should be the average of those corresponding to the two bands. In our case, a molecular weight of 95,000 could be the weight of the monomeric structure, but because under certain conditions (KCl or mercaptoethanol treatment) the 75,000 daltons form disappears, the existence of two different monomeric structures one of 75,000 and another one of 100,000 could be possible.

There is the possibility, however, that the 100,000 daltons form is derived from the 75,000 by the addition of some small component. Rosenberry (1975b) arrived at a value of 76,000 gr/mole of active sites for the equivalent weight of Electric eel AChE. After incubation at 37° for 24 h, forms with molecular weights of 64,000, 49,000 and 39,000 were detected, which all showed enzyme activity. These results suggest that small fragments of the protein structure can be detached by the action of endogenous

proteolytic enzymes, without affecting the catalytic activity of the enzyme, so these fragments have to be remote from the catalytic subunit. A 39,000 daltons subunit is however half the proposed monomeric structure weight and this agrees with the suggestion of Vigny et al (1978) that the size of the catalytic polypeptide chain (in the range of 60,000-80,000) could be in fact much larger than what appears necessary for the existence of catalytic site, because according to Hogber-Raibaud and Goldberg (1977) the elementary globular organized unit seems to be only 10 fold smaller. Thus, they suggest, that the largest part of the polypeptide chain might well not be necessary for catalytic purposes but might be involved in structural interactions with synaptic elements, including, for instance, the tail of the electric organ enzymes.

In most of the preparations, forms with molecular weights of 150,000, 167,000, 200,000 and 230,000 appear and they might correspond to dimer structures of 75,000-100,000, and because these forms disappear after KCl treatment some type of electrostatic interaction might contribute to their formation. A 150,000 molecular weight form has been reported by Wenthold et al (1974), accounting for as much as 60% of the rat brain enzyme. Finally forms with quite high molecular weights in the range of 350,000-450,000 were detected in all preparations, representing different aggregation states of the monomeric structure or structures. In every case, the form of 400,000-450,000 was particularly intense and it was not affected by any treatment or coriditions of the samples. It seems quite probably that this structure could represent the tetrameric

form of the enzyme, and the possibility of a heterogenous composition (100,000 and 75,000 subunits) could be present. When the samples were stored in the deep-freeze for a long time, forms of 550,000, 600,000 and even 700,000 were detected, representing a much higher degree of aggregation. Rieger and Vigny (1976) have calculated a molecular weight of 435,000 for the 10S enzyme from rat brain and if the 4S had a weight of 115,000, the medium molecular weight forms should have 7-8S of sedimentation coefficient which it agrees with the comments on sucrose gradient experiments.

## G. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

The purified preparations treated with mercaptoethanol and SDS produced the following bands after the application of this technique: 150,000, 75,000, 50,000, 38,000, 33,000 and 30,000. Thus 75,000 could represent the monomeric structure. However, because forms of very similar weight were detected after incubation of the enzyme at high temperature, it could be possible that the monomeric polypeptide chain does not contain any disulfide bridges. The polypeptides of 75,000, 50,000, ~40,000 and ~30,000 seem to represent the result of a progressively cleavage of the chain with probably very little effect on the catalytic site. Further experiments are needed to completely formulate a pattern for the structure of acetylcholinesterase from rat brain.

## H. EPILOGUE

The main aim of this study was an attempt to determine the nature of the multiple molecular forms of acetylcholinesterase from rat brain and the problem has been examined from three major viewpoints:

1. How the enzyme can be effectively solubilized.
2. The effect of the solubilization procedure on kinetic and thermodynamic behaviour of the enzyme.
3. The effect of the solubilization procedure on the number of multiple molecular forms.

As a corollary to these three questions, there is a further problem related to the way that the enzyme is bound to the membrane and the physiological significance of that type of binding.

From the work of this thesis, the solubilization efficiency of dilute buffer, Triton and papain have been discussed and from these experiments, it seems that the enzyme is a peripheral protein, where polar groups of the membrane (sialic acid, aminoacid or polar lipids residues) can interact with the polypeptide structure of the enzyme. The different multiple forms appear to behave in a similar way when subject to kinetic studies suggesting a very similar active site environment in all of them. Finally it seems quite probable that the released forms of the enzyme are almost the same and are rather independent of the extraction procedure. Therefore, for extension to mammalian brain enzyme, it can be concluded that probably the membrane enzyme interaction is much simpler than was thought. However, further

investigations need to be carried out to establish the tertiary and quaternary structure of the enzyme and the precise physiological significance of the multiple forms of acetylcholinesterase.

# SECTION V

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# BIBLIOGRAPHY

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The lysosomal membranes showed two peaks of phospholipase activity with optima at pH4.5 and 7.7 (Fig. 2). The incorporation of 1mM-EDTA instead of 10mM-$CaCl_2$ in the reaction medium decreased the neutral phospholipase by one-half, but increased the acid phosphatase 4-fold. The neutral phospholipase is thus similar to that reported in rat liver lysosomes (Waite *et al.*, 1969; Colbeau *et al.*, 1974).

The lysosomal membrane preparation also contained triacylglycerol lipase activity and acylglycerol lipase activity with distinctly acid pH optima and little or no activity at pH7.7 (Fig. 2). Furthermore, these activities were increased by 1mM-EDTA over the whole pH range where they were active.

Lysosomal membranes therefore contain a phospholipase active at a pH close to neutrality (pH7.7), dependent on calcium and distinct from acid phospholipase, triacylglycerol lipase and acylglycerol lipase activity.

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## Multiple Forms of Acetylcholinesterase from Fresh and Toluene-Stored Rat Brains

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Acetylcholinesterase is a membrane-bound enzyme and most work on this enzyme has been carried out after bringing it into solution. However, the extraction procedure affects the degree of solubilization and in some cases the multiple molecular forms (McIntosh & Plummer, 1973; Dudai & Silman, 1974). During the preparation of acetylcholinesterase, tissues may be stored in toluene for several months before processing by using the method of Rothenberg & Nachmansohn (1947). It was therefore decided to see what effect this pretreatment had on the yield and purification of acetylcholinesterase and also on the number and distribution of the multiple molecular forms.

### *Experimental*

*Solubilization of acetylcholinesterase from fresh rat brains.* Brains from freshly killed rats were extracted with 30mM-sodium phosphate buffer, pH7, and then centrifuged at 100000*g* for 1h. The acetylcholinesterase activity in the supernatant was then determined by a pH-stat method (McIntosh & Plummer, 1973) and this was referred to as the naturally soluble form of the enzyme. The pellet was resuspended in 1% (w/v) Triton X-100 in 30mM-phosphate buffer and again centrifuged: the supernatant was then assayed for acetylcholinesterase activity.

*Toluene treatment of rat brains.* Alternatively, the brains were removed from the rats immediately after death and stored in dry toluene for 3 to 6 months at 4°C. At the end of this time, the brains were removed from the toluene and placed in a Petri dish in the fume chamber until the residual toluene had evaporated. The acetylcholinesterase was then extracted from the brains with buffer and detergent as described above.

The protein content of the enzyme extracts was determined by a biuret method (Plummer, 1978).

*Polyacrylamide-gel electrophoresis.* Samples of the extracts were subjected to electrophoresis on slabs containing a concave gradient of polyacrylamide from 4 to

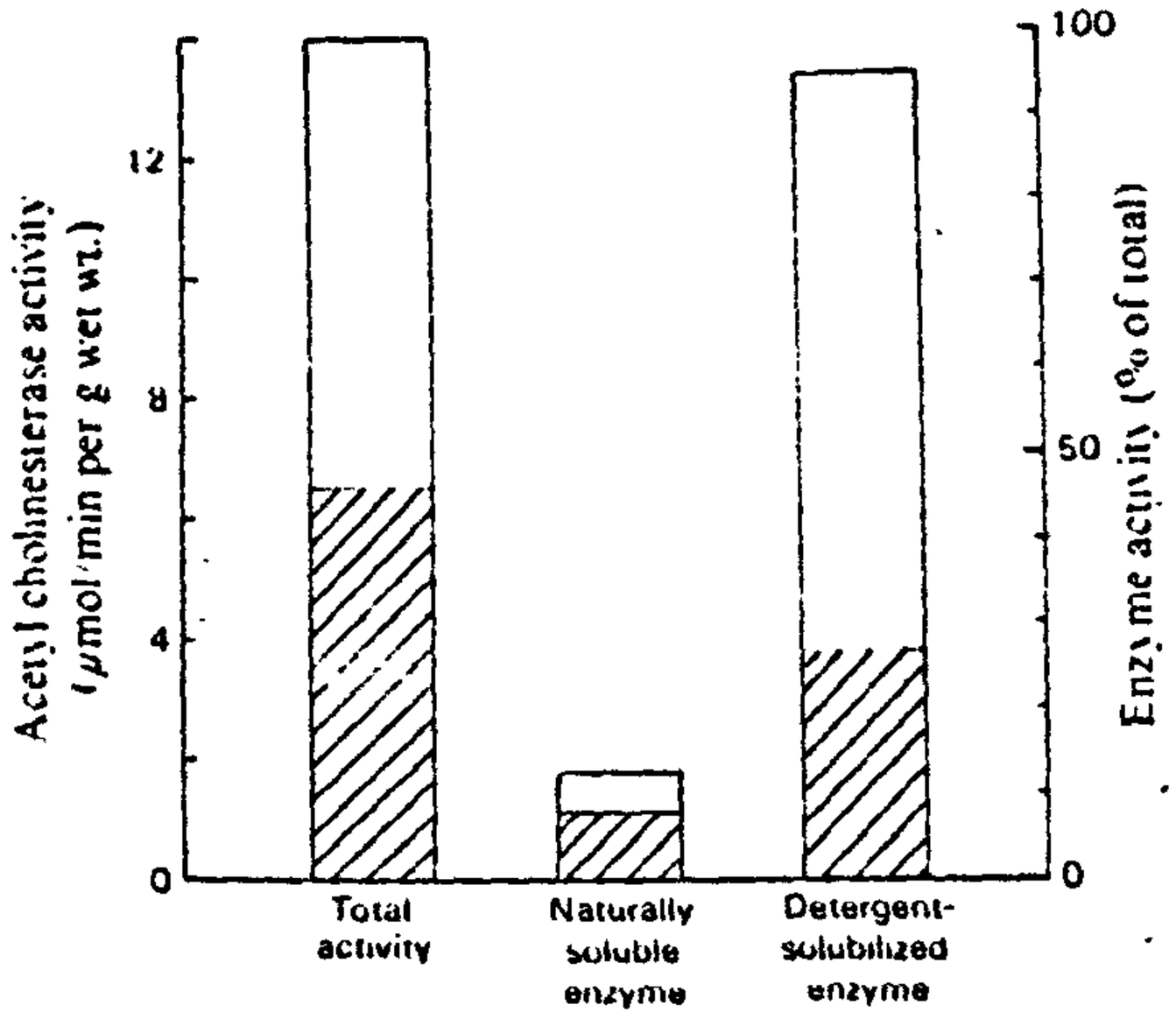


Fig. 1. *The effect of the extraction procedure on the activity of acetylcholinesterase obtained from rat brains*

□, Fresh rat brains; ▨, toluene-stored rat brains. Experimental details are given in the text.

26%. Zones of acetylcholinesterase activity were visualized by incubation with acetylthiocholine as substrate (McIntosh & Plummer, 1973). When ethopropazine (30 $\mu$M) or eserine (10 $\mu$M) was included in the incubation medium, the gels were soaked in a solution of the inhibitor for 1 h before incubation.

*Results and discussion*

In the case of fresh rat brains, about 13% of the acetylcholinesterase activity was soluble in dilute buffer solution and the remainder was readily solubilized by Triton X-100. Storage of the rat brains in toluene did not increase the amount of acetylcholinesterase brought into solution by extraction with dilute buffer (Fig. 1). Triton

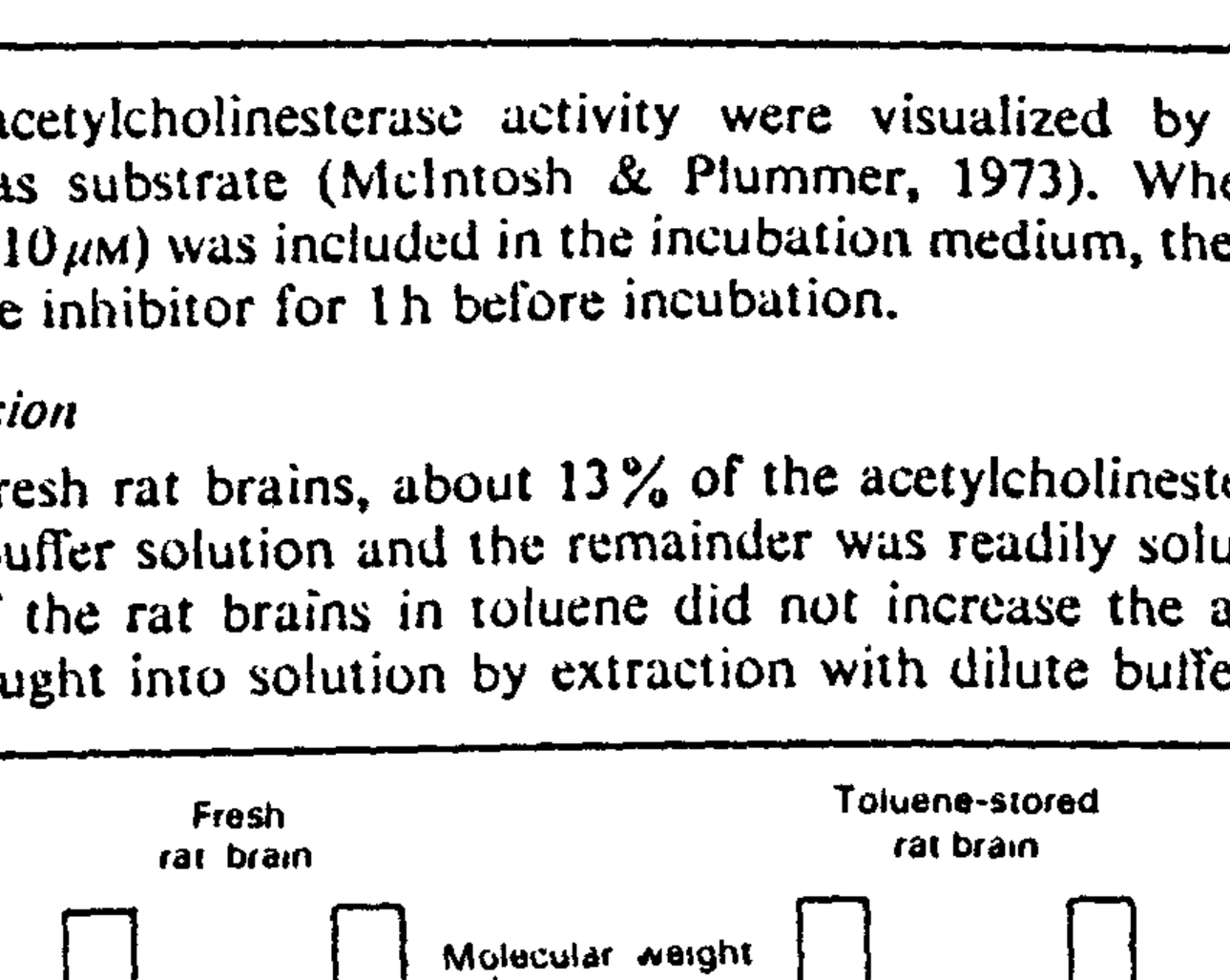


Fig. 2. *Gradient polyacrylamide-gel electrophoresis of acetylcholinesterase preparations from rat brain*

Depth of staining was: ■, strong; ▩, moderate; ▨, light; ⁞⁞, faint. Experimental details are given in the text.

X-100 was still needed to solubilize the enzyme although the activity obtained was less than that with fresh rat brains. The toluene treatment was not very effective at removing unwanted protein since the specific activity of the Triton-solubilized enzyme was the same at 0.34 $\mu$mol/min per mg of protein whether the brains were from freshly killed animals or had been stored for several months in toluene. Furthermore, the toluene treatment would seem to make it more difficult to solubilize the enzyme since only 75% of the total activity present in the brain after toluene treatment could be solubilized whereas 100% of the activity present in fresh brains could be brought into solution. There seems therefore little point in storing the brains in toluene.

The toluene treatment could also affect the multiple forms of acetylcholinesterase and this was the next question investigated by subjecting the extracts to gradient polyacrylamide-gel electrophoresis.

A range of molecular weights was obtained for the naturally soluble and detergent-solubilized enzyme from 75000 to 440000 (Fig. 2) and it is tempting to suggest that this represents a range of oligomers (McIntosh & Plummer, 1973). Incubation with 10 $\mu$M-eserine caused the complete disappearance of bands 1, 2, 4, 5, 6 and 8, whereas a faint stain was obtained in the region of bands 7 and 9. The zones of activity were therefore due to cholinesterase activity, although some non-specific esterase activity appeared to be present in bands 7 and 9. Incubation with 30 $\mu$M-ethopropazine made no difference at all to the electrophoretic pattern, showing that the bands were due to acetylcholinesterase activity (EC 3.1.1.7) and not a non-specific cholinesterase (EC 3.1.1.8).

Similar, but not identical, electrophoretic patterns were obtained for the toluene-treated brains, although bands 8 and 9 with mol.wts. of 350000 and 440000 were missing from both the buffer and detergent extracts. Furthermore, the low-molecular-weight zones (bands 1–3) were absent from the toluene-treated brains that were then extracted with dilute buffer.

Toluene therefore does appear to modify the molecular forms obtained from rat brain, emphasizing once again the need to specify the methods and conditions of solubilization when reporting on the molecular species of acetylcholinesterase obtained from a tissue.

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## The Purification and Reconstitution of the Mitochondrial Adenine Nucleotide Translocase of *Saccharomyces cerevisiae*

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The adenine nucleotide translocase is a transport system catalysing the exchange of either ADP or ATP across the inner mitochondrial membrane (Pfaff & Klingenberg, 1968). It makes mitochondrially generated ATP available to the cytoplasm, and consequently, a detailed understanding of this transport system is important in the understanding of cellular energetics.

We report the purification of the adenine nucleotide translocase from the mitochondria of *Saccharomyces cerevisiae* strain L410 ($\alpha$, *ura*, *his*), and the reconstitution of its biological activity in phospholipid vesicles. The purification and reconstitution procedures used are basically those of Kramer & Klingenberg (1977), the mitochondria